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Yu, Nana

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Nana Yu

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by

Nana Yu

born on 21 June 1985
in Shandong, China

Supervisor
Prof. J.T.M. Elzenga

Assessment Committee
Prof. J.D. van Elsas
Prof. R. Pierik
Prof. J.H.C. Cornelissen

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Chapter1

General introduction

Roots play vital roles in growth and development of plants. They function in uptaking of water and nutrients in the soil, in the interaction with symbiotic fungi and bacteria, in carbohydrates storage, and in maintenance of the rhizosphere (Zhu et al. 2011; Petricka et al. 2012; Scheres et al. 2002; Morris and Walker 2003). Environmental conditions greatly affect plant development and productivity. As plants cannot escape from adverse environmental conditions, they utilize autologous mechanisms to cope with environmental stresses. Among these mechanisms, receptor-like kinases (RLKs) localized in the plasma membrane of plant cells, have become the focus of more and more studies on signal perception and transduction in various aspects of plant growth and development. Moreover, many identified RLKs are also found to respond to environmental cues and trigger acclimation to cope with different biotic and abiotic stresses.

Arabidopsis thaliana has been widely used in signal transduction studies since the 1980s because of it's short life circle and simple genome model for plant physiological and genetic analyses (Smith and De Smet 2012; Osmont et al. 2007; DeYoung and Clark 2008; Pé ret et al. 2009). Since the first plant receptor kinase, the maize putative protein kinase-encoding cDNA clones (*ZmPK1*), was reported in maize (Walker and Zhang 1990), more than 610 members of RLK genes have been identified in *Arabidopsis*, representing nearly 2.5% of all *Arabidopsis* protein coding genes (Shui and Bleecker 2001a). Unlike receptor tyrosine kinases (RTKs) found in animals, which contain the tyrosine kinase catalytic domain, plants have the serine/threonine signature, which is structurally related to the receptor tyrosine kinases (Walker and Zhang 1990; Castells and Casacuberta 2007). Based on the structure of the extracellular domain, all RLK genes are divided into three groups. The transmembrane RLKs represents the largest group with more than 400 members, which have a typical structure comprised of a signal peptide, an extracellular domain, a serine/threonine transmembrane domain, and a cytoplasmic kinase domain. The second group, the receptor like-cytoplasmic kinase (RLCK) family, which lacks the extracellular domain, has 135 members (Shui and Bleecker 2001). The third group, with 56 members, is the receptor-like proteins (RLPs) which lacking a cytoplasmic domain (Wang et al. 2008).

The extracellular domains of RLKs are highly diverse. Based on the similarity of these domains, RLKs are classified into more than 21 subfamilies, among which the Leucine-rich repeat kinases (LRR-RLKs) represents the largest group in *Arabidopsis* with more than 200 members (Shiu and Bleecker 2001b). The extracellular LRR motif has a stretch of around 20-29 amino acids with conserved hydrophobic leucine residues with the consensus sequence of LxxLxLxxNxL or LxxLxLxxCxxL, form a short B-strand (Kobe and Deisenhofer 1994; Kobe and Kajava 2001). In this consensus sequence, "x" represents the non-conserved residues, while "L" represents Leucine, Isoleucine, Valine or Phenylalanine. "N" is Asparagine, Threonine, Serine, or Cysteine, and "C" is Cysteine, Serine or Asparagine. The most common length for an LRR is 24 residues, but repeats containing from 1 up to 32 residues can also be found in the extracellular domain (Matsushima and Miyashita 2012; Matsushima et al. 2010). Based on the amino acid sequence similarity between kinase domains, the

LRR-RLKs can be subdivided into 14 subgroups, LRR I to XIV (Shiu and Bleecker 2003). In *Arabidopsis* there are 223 LRR-RLKs, but only about 60 have been functionally described to date (Wu et al. 2016). Most of these characterized LRR-RLKs are assumed to be involved in protein-protein interactions whereas other motifs are implicated in binding to various carbohydrate substrates. An exceptional type of substrate in implicated for the LRR-RLK, BRASSINOSTEROID INSENSITIVE1 (BRI1), which may binds directly to a steroid hormone (Wang et al. 2001; She et al. 2011).

Receptor-like kinases in *Arabidopsis* root system architecture

Although the root system architecture varies among different species and can be modulated by the conditions encountered in the soil environment, the basic root system morphology is controlled by the inherent genetic blue print (Osmont et al. 2007). The *Arabidopsis* seedling displays a typical root system for dicotyledons, consisting of one primary root (PR) that formed during embryogenesis, lateral roots (LRs) branching out from PR, and root hairs (RHs) that originate from PR epidermal cells (Barrada et al. 2015). In the *Arabidopsis* primary root, a slowly dividing stem cell pool of initial cells surrounds the quiescent center (QC), with three to four infrequently dividing cells. The division of initial cell gives rise to the files of distinct cell layers (tissues), including the epidermis, the cortex, the endodermis, the pericycle and the stele that surrounds the vascular bundels (**Figure 1**). The longitudinal axis of the PR demonstrates a developmental time line: within the apical meristem zone, initials and their daughter cells divide multiple times producing similar sized daughter cells, while in the transition zone only a few cells still divide and the majority of cells start to elongate. Cell size in the elongation zone increases sharply, compared with those cells in the transition zone, until they have reached their final length. In cells that have reached their final size, polarized cell enlargement leads to the formation of root hairs, demarcating the distal margin of the maturation zone of a root.

Embryo receptor like kinases

The *Arabidopsis* zygote undergoes an asymmetric division to generate a smaller apical cell and a larger basal cell. The apical cell-lineage generates an eight-cell embryo proper with an apical domain (AD) and central domain (CD) after a series of divisions. The AD generates the cotyledon and the shoot meristem, whereas the CD produces part of the cotyledon, the hypocotyl and the root meristem initials (Mayer et al. 1991; Jürgens et al. 1991; Slane et al. 2014; Meinke 1991). The basal cell produces the suspensor that plays an important role during embryo development, including (i) pushing the embryo proper into the endosperm cavity, (ii) transport of molecules involved in nutrition and growth regulation and (iii) biosynthesis of plant hormones (Kawashima and Goldberg 2010).

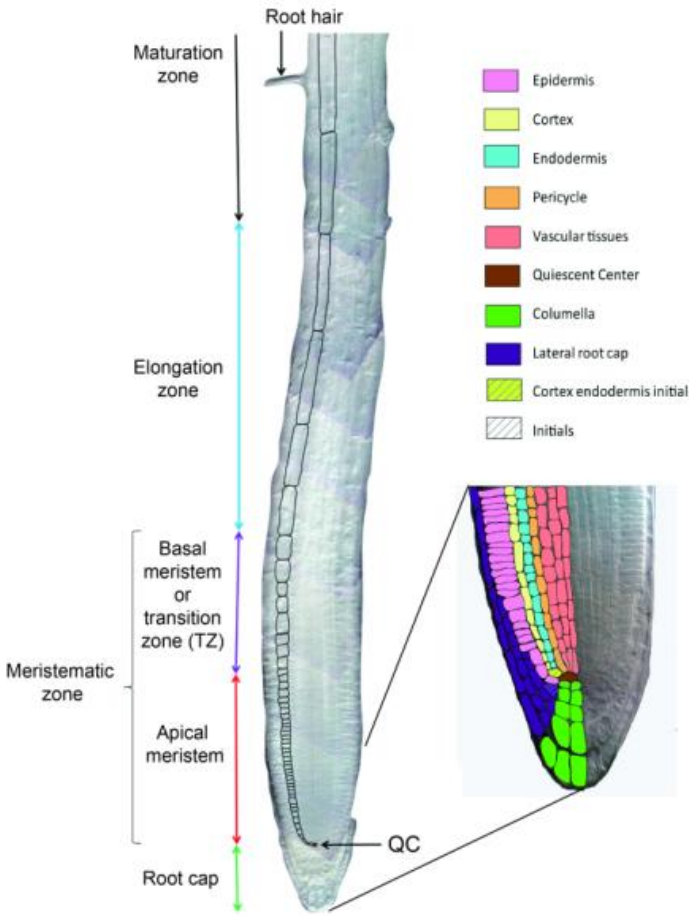

Figure 1. The longitudinal axis and root radial patterning of primary root of the model flowering plant *Arabidopsis thaliana*. The primary root tip of wild-type Columbia is consisted by three different zones, including the meristematic zone, the elongation zone, and the maturation zone. The black contours of cortical cells highlight the increase of cell size (left) and each color represents a different cell layer (right). Figure modified from Barrada et al. (2015).

The development of the plant embryo is a complex process and experimental evidence indicates that RLKs play major roles in the intercellular signaling in the embryo development (**Figure 2**, reviewed by Nodine et al. 2011). The length of the suspensor determines the speed of the development progression of the embryo in *Arabidopsis*, and the SHORT SUSPENSOR (SSP) gene was the first RLCK identified to functioning in the zygote (Bayer et al. 2009; Babu et al. 2013). Mutants lacking a functional SSP gene fail to generate and elongate basal cells, resulting in a short suspensor phenotype. Genetic analysis suggests that SSP acts upstream of the YODA (YDA) MITOGEN-ACTIVATED PROTEIN (MAP) kinase cascade, which is required for partitioning of the embryo and determine the extra embryonic fates

(Lukowitz et al. 2004), but SSP regulates this pathway through a unique parental-original effect (Bayer et al. 2009). In addition, the SSP is found related to another group of RLCKs, the BRASSINOSTEROID-SIGNAL-KINASES (BSKs), which *in vitro* are phosphorylated by BRI1 and which *in vivo* interact with BRI1 and regulate cell elongation in *Arabidopsis* (Tang et al. 2008). However, no direct evidence was found that SSP acts in the BRI1 pathway of controlling nuclear gene expression and embryo development.

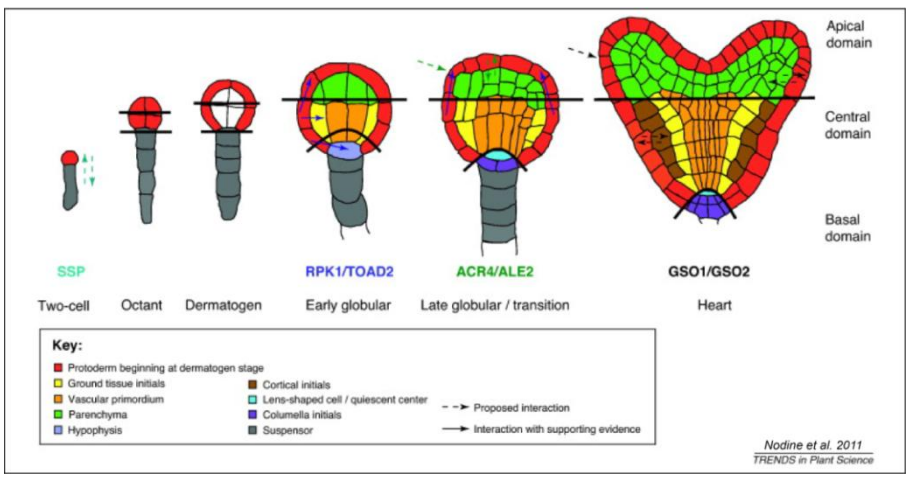

Figure 2. RLK and RLCK functions during embryogenesis of *Arabidopsis*. Dotted arrows represent possible cell interaction and arrows with solid lines represent interactions that have been confirmed by experiments. SSP acts as a signal to promote elongation and asymmetric division of zygote via the YDA pathway MAP kinase cascade to regulate basal cell development. RPK1/TOAD2 are redundantly required for maintaining protodermal cell fate identity during the early globular stage of *Arabidopsis* embryo development. At the later globular and transition stages, ACR4/ALE2 positively regulate protoderm gene expression and the integrity of protoderm in the apical domain, which is required for normal cotyledon emergency. GSO1/GSO2 are important for maintaining epidermal function from heart stage of the embryo development. Figure adapted from Nodine et al. (2011).

In the early globular stage of *Arabidopsis* embryo development two closely related RLKs, RECEPTOR PROTEIN KINASE1 (RPK1) and TOADSTOOL2 (TOAD2), are required for the maintenance of protodermal cell fate identify in the central domain (reviewed by Nodine et al. 2011). The localization of RPK1 and TOAD2 translational fusions of green fluorescent protein (GFP), together with the cell specific markers in toadstool embryos, strongly indicates that RPK1 and TOAD2 are redundantly required for *Arabidopsis* embryonic pattern formation. They are also required together for cotyledon initiation during later embryonic stages (Nodine and Tax 2008).

In *maize*, the CRINKLY4 (CR4) gene was found to encode a TNFR-like receptor-like kinase that is involved in the leaf epidermis differentiation (Becraft et al. 1996). *Arabidopsis thaliana* homologue of CR4 (ACR4), a putative receptor-like kinase

receptor of *Arabidopsis*, is homologous to the maize CR4 gene and required for proper development of the embryo (Tanaka et al. 2002). At the globular stage during early embryogenesis, ACR4 transcripts accumulate in both protoderm and inner cells at comparable levels, but change at the heart stage with relative higher level in the protoderm than in the inner cells, suggesting a role in epidermis differentiation. In addition, the ABNORMAL LEAF SHAPE1 (ALE1) and the ABNORMAL LEAF SHAPE2 (ALE2) that results in defects of cuticle formation, were found to act together with ACR4 during early stages of embryogenesis (Tanaka et al. 2007; Tanaka et al. 2001). These three genes play partially overlapping roles in positively regulating protoderm-specific gene expression and the formation of cotyledon through different modes of intercellular communications.

In addition, two other LRR-RLKs, GASSHO1 (GSO1) and GASSHO2 (GSO2) are essential for the normal development of the epidermal surface in *Arabidopsis* embryos (Tsuwamoto et al. 2008). Embryos of the double mutant GSO1/GSO2 display reverse bending of the embryo compared with the wild type embryo at the heart-torpedo transition stage. No difference was apparent between wild type and the GSO1/GSO2 double mutant embryos at the early heart stage, but in the mutant the apical part of the embryo will stick to the peripheral tissue of the endosperm, which is caused by abnormal development of the epidermis.

Based on microarray datasets from Keith Lindsey's group (Spencer et al. 2007) and John Harada-Robert Goldberg's microarray data (NCBI GEO: GSE12404), more than 300 expressed receptor-like genes were detected during different stages of embryogenesis (Nodine et al. 2011). Apart from high expression of seven RLKs (SSP, RPK1, TOAD2, ACR4, ALE2, GSO1, and GSO2) discussed above, a large number of RLKs and RLCKs with known functions during adult growth and development were detected during embryogenesis, including BARELY ANY MERISTEM 1/2 (BAM1/2) (DeYoung and Clark 2008), SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1/2 (SERK1/2) (Fan et al. 2016; Albrecht et al. 2005) that is involved in post-embryo development, BRI1 and BSKs (Tang et al. 2008) that are involved in the brassinosteroids signal transduction pathway, and RLK PEP1 RECEPTOR1 (PEPR1) (Yamaguchi et al. 2006) and the RLCK AVRPPHB SUSCEPTIBLE1 (PBS1) (Swiderski and Innes 2001), with known functions in pathogen defense responses. Thus, signaling via RLKs and RLCKs is important during this part of the life cycle of a plant, and there are still a large number of RLKs and RLCKs that are expressed during embryogenesis, but for which a specific function during embryonic pattern formation still has to be established.

Root apical meristem maintenance RLKs

Primary meristems of shoot and root are initiated during embryogenesis and control plant growth along the main body axis. The CLAVATA pathway that acts in the shoot apical meristem (SAM) to control shoot and floral meristem size in *Arabidopsis* is a good illustration of study on the role of RLKs in signal transduction

(**Figure 3**). In the SAM of *Arabidopsis* the central zone (CZ), which is surrounded by the peripheral zone (PZ), consisting of three clonal layers: the epidermis layer (L1), the sub-epidermis layer (L2), and the interior bulk of the meristem (L3). CLAVATA1 (CLV1) contains an extracellular domain with 21 leucine-rich repeats (LRRs) and is expressed in and around the organizing zone (OZ) which underlies the L3 cell layer of the shoot meristem (Clark et al. 1993). CLV1 forms together with CLV2 the receptor kinase complex that binds CLV3, a ligand that belongs to the large CLAVATA/ENDOESPERM SURROUNDING REIGON (CLE) gene family (Clark et al. 1995). The secreted ligand CLV3 is expressed at the apex of the SAM and moves to the CLV1-expressing cell in the L3 cell layer, where the activated complex causes down-regulation in the OZ of WUSCHEL (WUS) which plays a central role in stem cell maintenance in the CZ (Clark et al. 1993; Stahl et al. 2009).

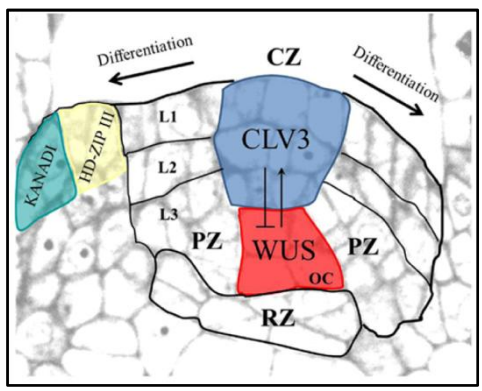

Figure 3. The CLAVATA pathway that acts in the shoot apical meristem of *Arabidopsis thaliana*. The shoot meristem contains a central zone (CZ), the peripheral zone (PZ), and the rib zone (RZ) as shown in the figure. In dicotyledoneae angiosperm, the shoot meristem is divided into three cell layers (L1 to L3) that contribute differentially to plant growth. The L1 and L2 layer cells divide predominantly to form the epidermis and sub-epidermis tissues, respectively. The cells in the L3 layer mainly give rise to the internal tissues by dividing to all directions. CLV3 serves as a negative feedback signal (shown in blue) that binds to several RLKs, including LRR-RLK CLV1 and RPK2/TOAD2, the LRR-RLP CLV2, and the RLCK CRN, to restrict the organizing center (OC) by down-regulating WUS transcription (shown in red). In the leaf primordium, adaxial and abaxial cell fates are marked by expression of HD-ZIP III (shown in yellow) and KANADI (shown in blue) family genes, respectively. Figure modified from Bosc á*et al.* (2011) and Groβ-Hardt and Laux (2003).

The root apical meristem (RAM), established during embryogenesis, comprises four types of initial cells and three to four infrequently dividing cells, the quiescent center (QC) (**Figure 4 A**) (Scheres et al. 2002). Cell division of these four sets of initial cells give rise to different layers of cells in the root apex, including the stele layer, the endodermis layer, the cortex layer, the epidermis layer, the columella, and the lateral root cap. The identity of the QC within the patch of stem cells in the RAM is specified by overlapping expression of the AP2 domain of PLETHORA (PLT) and the GRAS family SGORT ROOT (SHR)/SCAREROW (SCR) transcription factor, mutants of which display arrested root growth (Petricka et al. 2012; Aida et al. 2004).

The QC is essential for maintenance of the undifferentiated state of stem cell initials and it regulates cell division in the columella by arresting cell differentiation of the columella initial cells (van den Berg et al. 1997). Roots of a putative mutant lacking expression of the homeobox gene WUSCHEL-RELATED HOMEOBOX5 (WOX5) display enlarge cells at the QC and columella stem cells (CSCs) positions, and starch granules (a feature of a mature columella cells) accumulate in stem cells other than the QC in the null allele mutant. These results indicate that WOX5 is required to prevent stem cell differentiation (Sarkar et al. 2007), similar to the role of WUS in the SAM (Stahl et al. 2009).

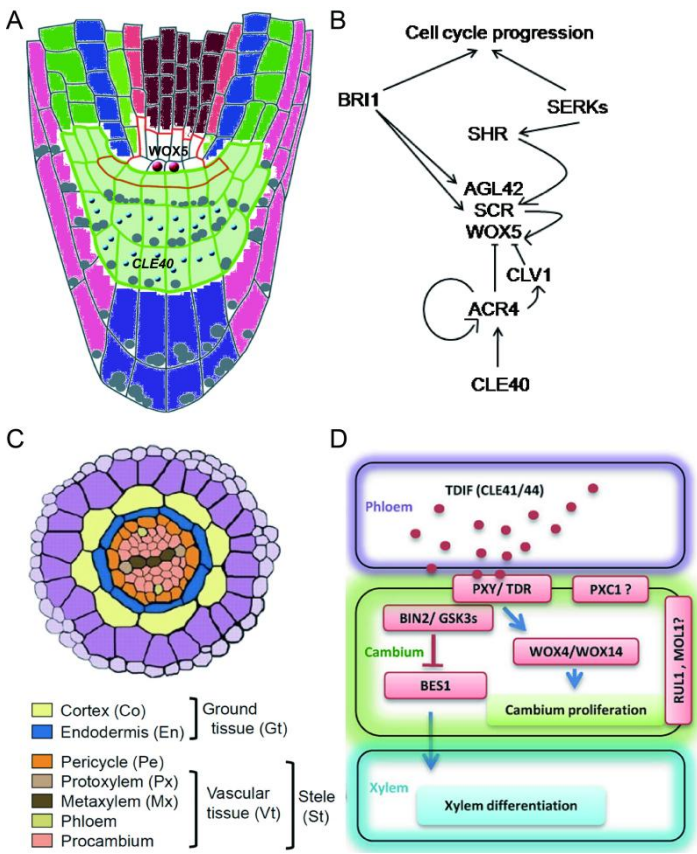

Figure 4. RLK functions during root apical meristem development and vascular development. A: Schematic of the root apical meristem of *Arabidopsis*. Colours represent different cell layers of root tip and the localization of the homeodomain transcription factor WOX5 (red) in the quiescent center, receptor like kinase ACR4 in the columella stem cell and columella cells, and it's ligand CLE (blue). (Figure modified from Stahl and Simon 2012). **B:** Conceptual model of regulation of root apical meristem through CLE peptide/RLK pathway. **C:** Schematic of transverse section of meristematic region of the *Arabidopsis* root (Figure modified from Miyashima et al. 2011). **D:** Conceptual model of regulation of vascular patterning through TDIF peptide signaling (Figure modified from Yang and Wang 2016).

Over-expression of CLV3 related members of the CLE families (CLE14, CLE19,

CLE20, and CLE40) results in arrested root growth, suggesting that the CLV-like signaling pathway also operates in the root apical meristem (Meng and Feldman 2010; Stahl et al. 2009; Stahl and Simon 2009). ACR4 is expressed in the outer cell layer of embryos and involved in proper embryogenesis (Tanaka et al. 2002). In the RAM of plants transformed with the HISTONE2B::YFP fusion protein encoding gene, ACR4 expression was observed in the QC, the columella initials and the columella cells below the QC, the lateral root cap and the initial cells that give rise to the epidermal tissue (Gifford et al. 2003). CLE40, which is also expressed in the embryo, also acts as a secreted ligand of the ACR4 receptor in the columella and the columella stem cells, where it up-regulates its own expression and that of CLV1, restricting WOX5 expression to the QC (Pallakies and Simon 2014) (**Figure 4 B**). Together, this indicates a WOX5-dependent mechanism of stem cell fate regulation by CLE40, CLV1, and ACR4.

In addition to the CLE peptide-receptor pathway, the LRR-RLK BRI1 was identified playing a specific role in the regulation of RAM through a steroid hormone-RLK pathway (González-García et al. 2011; Hacham et al. 2011). The BRI1 gene encodes a widely expressed putative receptor of the hormone brassinosteroid (BR), which modulates cell elongation and division throughout growth and development of a plant (Clouse et al. 1996; Li and Chory 1997). The *bri1* mutant displays a severely dwarfed phenotype and can't be rescued by BRs treatment. Generally, treatment with the brassinosteroid brassinolide (BL) promotes primary root growth at low levels and inhibits growth progressively at higher levels (Clouse et al. 1996). Both gain- and loss-of function of BR-related *Arabidopsis* mutants possess a reduced meristem size indicating a possible role for BRs in optimal root growth. In fact, BRs act on the root stem cells by promoting the QC cell renewal and controlling the cell cycle progression and differentiation necessary for maintaining the meristem size (González-García et al. 2011). In addition, 4 nM BL treatment of the *bri1* mutant causes increased expression of WOX5 and SCR, and the lack of SHR and WOX5 expression in the *serk* triple mutants (Du et al. 2012; Gou et al. 2012), indicating that RLKs are candidate molecules to function as receptor or co-receptors in the regulation of root apical meristem maintenance.

Stele and ground tissue RLKs

The vasculature of *Arabidopsis* root is organized into a central stele, comprised of the xylem and phloem, and is formed in the RAM by initial cells that give rise to the protoxylem, metaxylem and procambial cells, depending upon the direction of cell division (**Figure 4 C**) (Ohashi-lto and Fukuda 2010; Scheres et al. 1994; Dolan et al. 1993). These tissues are originally formed from a set of pericycle/vascular initials proximal to the QC, and they grow symmetrical along a central axis with protoxylem at the poles and metaxylem in the center (Zhang et al. 2011).

The BREVIS RADIX (BRX) gene family of *Arabidopsis* is a class of transcription factors that control growth and development throughout the plant and of which *BRX* is the only gene that has a role in root system development of *Arabidopsis* (Mouchel

et al. 2004). BRX is expressed in the vasculature and the reduced root size phenotype of *brx* mutant results from the reduced expression of a rate-limiting enzyme in brassinosteroid biosynthesis pathway (Mouchel et al. 2006). Expression of *BRX* is strongly induced by auxin and mildly repressed by brassinolide, indicating that *BRX* mediates a feedback loop between brassinosteriod and auxin signaling enabling optimal root growth. Recently, the LRR-RLK gene, BARLY ANY MERISTEM3 (BAM3) was identified as suppressor of root meristem growth and protophloem development defects of *brx* mutant (Depuydt et al. 2013). While CLE45 treatment severely inhibits root meristem growth in wild type roots, the roots of the *bam3* mutant are insensitive to application of the CLE45 ligand. As expression of *bam3* is increased in both *brx* mutants and roots treated with CLE45 peptide, protophloem differentiation in the transition zone of the root tip is caused by activation of BAM3 binding to CLE45, and BRX promotes protophloem differentiation through inhibition of BAM3 expression.

In the *Arabidopsis* genome, thirty-two CLE genes have been identified to be involved in many aspects of biological processes of plant growth and development (Betsuyaku et al. 2011; Jun et al. 2008). Among them, CLE41 and CLE44 encode a 12-amino acid TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) peptide. In the vascular meristem, the LRR-RLK PHLOEM INTERCALATED WITH XYLEM/TDIF RECEPTOR (PXY/TDR), which shares high level sequence similarity with CLV1, perceives the TDIF signals from phloem to regulate the undifferentiated procambial cell fate during secondary growth (**Figure 4 D**, Hirakawa et al. 2008; Ohyama et al. 2008; Yang and Wang 2016). The TDR, localized in procambial cells, is activated by TDIF and then promotes cell division of procambial cells and suppresses differentiation of the procambial cells into xylem cells (Hirakawa et al. 2010). Additionally, expression of WOX4 increases in the presence of TDIF in a TDR-dependent manner (Hirakawa et al. 2010). A mutation in TDR causes both the suppression of procambial cell proliferation and the enhancement of xylem differentiation, whereas a mutation in WOX4 only suppress the proliferation of procambial cells, suggesting that TDIF-TDR signaling regulates vascular stem cell fate by two independent pathways that appear to diverge early after TDIF recognition.

Several other RLKs are also implicated in the development of the vascular system. For instance, two members of the BRI1 family of plant steroid receptors, BRI1-LIKE1 (BRL1) and BRI1-LIKE1 (BRL3), are predominantly expressed in the vascular tissues and function specifically in provascular differentiation to maintain xylem and phloem (Caño-Delgado et al. 2004). Another RLK, XYLEM INTERMIEXD WITH PHLOEM1 (XIP1), displays an aberrant accumulation of highly lignified cells and phloem cells adjacent to xylem cells in stem sections, similar to the *pxy* mutant phenotype, indicating that XIP1 plays a role in differentiation of phloem cells in vascular development (Bryan et al. 2012). Moreover, MORE LATERAL GROWTH1 (MOL1) and REDUCED IN LATERAL GROWTH1 (RUL1) were identified as opposing regulators of lateral expansion of

plant growth axes, and they might function to recognize and communicate long or short range signals to cambium cells (Agusti et al. 2011).

Regulation of epidermal cell fate and root hair formation

Root hairs are long cylindrical extensions of epidermal cells, and they are responsible for uptake of nutrients, establishing plant-microbe interactions, and helping plant anchoring to soil (Grierson et al. 2014). In *Arabidopsis*, epidermal cells are divided into two groups, the root hair cells that can produce root hairs and the non-hair cells, which lack root hairs. The *Arabidopsis* root epidermis is generated from a set of epidermal/lateral root cap initial cells formed during embryogenesis, and these initial cells can give rise to epidermal cells and cells of the lateral root cap, in the proximal and distal direction of the QC, respectively (Petricka et al. 2012). Like in many other members of the Brassicaceae, the epidermis of *Arabidopsis* possesses a distinct position-dependent pattern of root hair cells and non-hair cells, and how the identity of a newly formed epidermal cell, differentiating either into a root hair cell or a non-hair cell, is established, has been studied extensively to understand the regulation of cell type patterning in plants (Grierson et al. 2014; Dolan et al. 1994; Galway et al. 1994).

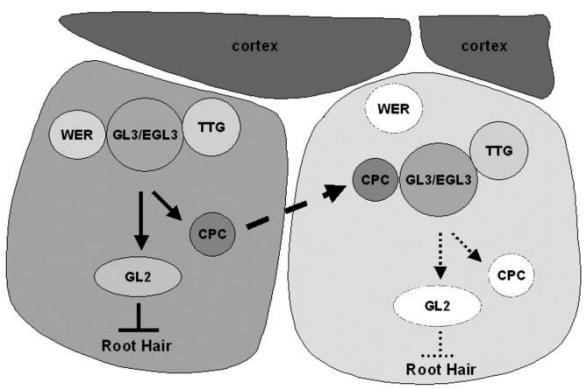

Figure 5. Control of epidermal root cell fate of *Arabidopsis*. In immature epidermal cells in the N position, the WER and MYB23 protein form an active complex with TTG and GL3/EGL3 proteins, and then positively regulate expression of GL2 and non-hair cell differentiation. However, the SCM pathway is proposed to negatively regulate WER transcription in the H position of the epidermal cell, which cause the GL3/EGL3 proteins succumb to the CPC/TRY/ETC1, which lead to inactive complexes, repression of GL2 and hair cell differentiation, in a position-dependent manner (Schiefelbein et al. 2009; Grierson et al. 2014). Figure modified from Bernhardt et al. (2003).

Several *Arabidopsis* mutants display a disrupted pattern of root epidermal cell types compared to wild type cells (Grierson et al. 2014). For instance, mutations in TRANSPARENT TESTA GLABRA (TTG), GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), and WEREWOLF (WER), alone or in combination, cause

plants to produce "hairy" roots, by changing non-hair cells in to root-hair cell (Galway et al. 1994; Bernhardt et al. 2003; Lee and Schiefelbein et al. 1999). On the other hand, CAPRICE (CPC), TRIPTYCHON (TRY), and ENHANCER OF TRY AND CPC (ETC1) are required for establishing the root-hair cell identity and mutation of these genes, alone or in combination, cause plants to produce "bald" cells at the former root-hair cell position (Simon et al. 2007; Wada et al. 1997; Kirik et al. 2004). Recently, an LRR-RLK SCRAMBLED (SCM) was discovered that enables immature epidermal cells to detect a positional signal and establish an appropriate cell-type pattern (Kwak et al. 2005). All these genetic findings to date led to a possible model for cell type pattern formation in the root epidermis of *Arabidopsis* (**Figure 5**) (Grierson et al. 2014).

Genetic analysis reveals discrete steps in the root hair development in *Arabidopsis* (Péret et al. 2009). In the initiation stage, rop proteins are first localized at the site where the root hair will be formed, before the hair begin to grow. Rop GTPases are localized to the tips of root hairs and control polar growth of *Arabidopsis* (Molendijk et al. 2001). Mutation of At3g51550, which encodes the FERONIA (FER) receptor-like kinase, induces sever root hair defects and reduced levels of active RAC/ROPs, indicating that FER assist in rop accumulation at the apical plasma membrane domains in the root tip (Duan et al. 2010). Within a minute after localized rop accumulation, the root hair cell wall begins to bugle out and the pH of the wall drops to pH 4 - 4.5, which is thought to activate expansion proteins that catalyze cell wall loosening (Grierson et al. 2014). As the bulge enlarges, large amount of endoplasmic reticulum and filamentous (F) actin accumulate in the developing swelling. In the tip growth stage the hairs grows to its final length by targeted secretion.

Lateral root development

In *Arabidopsis*, lateral roots are derived from the pericycle layer deep within the differentiation zone of the primary root (De Smet 2012). The mature pericycle cells along the xylem pole are stimulated to proliferate and re-differentiate into lateral root primordia (LRP), which contain their own meristems when they mature (Malamy and Benfey 1997). Histological studies showed that initiated LRP can then mature through eight stages (stage I-VII and emergence) defined by specific anatomical characteristics and cell divisions (**Figure 6 A**) (Malamy and Benfey 1997). Stage I of LR development begins with increased anti-clinal (perpendicular orientation to the root axis) divisions of cells in the pericycle layer. In stage II peri-clinal divisions have led to an outer and an inner layer and further peri-clinal divisions in the out layer result in a three layers primordium (outer layer1, outer layer2 and inner layer) in stage III. A second round of peri-clinal divisions in the inner layer creates a four-cell layer structure (layer1, outer layer2, inner layer1 and inner layer2). The LRP then penetrates the parent endodermis at stage IV, the cortex by stage V and the epidermis by stage VI. At stage VI and VII, the organization of the LRP shows similarity to the primary root tip, with epidermis, cortex, endodermis layers surrounding the stele and a root cap at the tip. Enlargement of the basal cells in the

outer layer1 promotes the increase in length of the LRP and finally the new lateral root emerges from the parent epidermis.

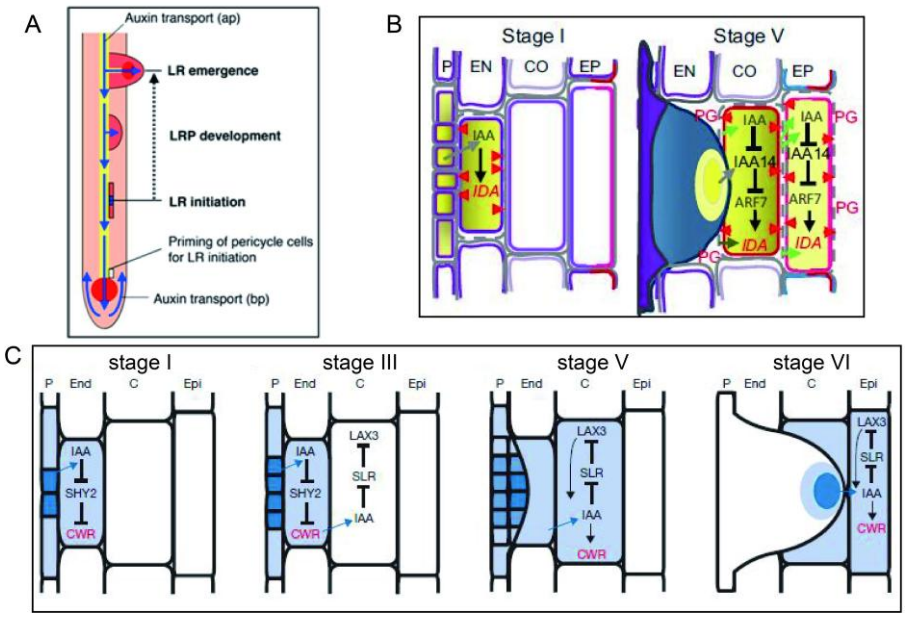

Figure 6. RLKs mediated pathways in the *Arabidopsis* lateral root development. A: Lateral root formation consists of three stages including LR initiation, LRP development, and LR emergence (Jung and McCouch 2013). **B:** Model of IDA-HAE/HSL2 signaling in lateral root emergency (Kumpf et al. 2013). **C:** Model for auxin-dependent lateral root emergency through auxin influx carrier LAX3 (Swarup et al. 2008).

During the development of LR auxin underpins each stage of the LRP development (**Figure 6**) (Nibau et al. 2008). Auxin is crucial for the determination of both the position and frequency of lateral root initiations and exogenous application of auxin can activate the whole pericycle to form LRPs (Himanen et al. 2002). A total of 1920 significantly differentially expressed genes were identified in auxin-activated pericycle cells and 15 potential key regulator genes were found associated with asymmetric cell division during lateral root initiation. Only one gene, At3g59420, was identified by all of the different filters, and this encodes a membrane localized receptor-like kinase ACR4 (De Smet et al. 2008). ACR4 is expressed in the small daughter cells after the first asymmetric cell division in the pericycle and mutants lacking ACR4 display additional cell divisions in the pericycle cell adjacent to the lateral root initiation site, an uncommon position for the lateral root meristems, or exhibit aberrant expression of the boundary marker (LBD5) and auxin response marker (DR5). Thus, ACR4 might be required for the autonomous specification of lateral root initials cell. As no ACR4 expression is observed in the neighboring pericycle cells, ACR4 signaling might prevent neighboring pericycle cells from being initiated as LRP, in a non-cell autonomously way. However, the mechanism of how ACR4 acts is not known.

In the single mutant of INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), HAESA (HAE), HAE-LIKE2 (HSL2) and in the *hae/hsl2* double mutant the density of LRs is significantly reduced compared with wild type, suggesting that these genes might play a role in LR development (**Figure 6 B**) (Kumpf et al. 2013). In *Arabidopsis*, abscission of the floral organ is controlled by the ligand peptide IDA through the receptor-like kinase receptors HAE and HLS2 (Cho et al. 2008). Emergence of new lateral root primordia depends on cell separation in the overlaying layers of the LR apex. At stage I and II, IDA expression in the overlaying cell layer is induced by auxin derived from the LRP which then binds to the HAE and HLS2 receptors located in the cell membrane. The activated receptors trigger expression of cell-wall-remodelling (CWR) genes and leads to cell wall separation in the endodermis. In the overlaying cortex and epidermal layer, both IDA and the receptor expression are coupled, in an ARF7-dependent manner, to the auxin influx carrier LAX3 (**Figure 6 C**) (Swarup et al. 2008). IDA signaling through HAE induces the expression of CWR enzymes to dissolve the cell walls and enable LR to penetrate the cortex and epidermis tissue from the deep xylem. Although IDA expression is 100-fold increased by exogenous auxin, the HAE and HSL2 receptors function as the limiting factor controlling cell separation (Kumpf et al. 2013). Thus, IDA-HAE/HSL2 signaling module is crucial for root/shoot cell separation during plant growth, but in different processes.

Other RLKs have been implicated in lateral LRP development and LR emergence as well. The double and triple mutant combinations of the TRANSMEMBRANE KINASE (TMK) subfamily of receptor-like kinases in *Arabidopsis*, *tmk1, tmk3* and *tmk4*, show a severe reduction in organ size and a related delay in growth stages (Dai et al. 2013). Moreover, they show reduced lateral root density and are insensitive to exogenously supplied auxin in root inhibition, suggesting that these RLKs might play a role in the auxin-mediated signaling pathway of lateral root emergence and development. In addition, other plant hormones also affect lateral roots development in a complicated network by controlling auxin synthesis and/or transport. In particular, BRs act synergistically with auxin to promote lateral root development through increasing auxin acropetal transport (Bao et al. 2004). In *bri1-119* background, the synthetic auxin-inducible promoter DR5 was severely decreased in the lateral root, compared to the wild type. This promotor was also decreased when treated with the BR biosynthesis inhibitor brassinozale, indicating that BRI1 is probably affecting lateral roots development in an auxin-dependent manner. However, currently there is not enough evidence to confirm the exact role of TMK, or that of the BRI receptor, in the process of LRP development.

RLKs involved in plant stress responses

Endogenous stimuli, such as plant hormones and ROS, modulate the molecular and biochemical mechanisms that increase the tolerance of plants to external stresses (Petricka et al. 2012; Potters et al. 2007; Overvoorde et al. 2010; Walter et al. 2009; Munns and Tester 2008). However, the external stress signals must first be perceived by plant cells or organs in order to initiate the acclimation to the new conditions. In

the case of molecular signals the perception is often by binding of the signaling molecule to a receptor protein located in the plasma membrane. RLKs play important roles in sensing the external stimuli and activating the down-stream elements of the signaling pathway via their serine/threonine kinase domains (Shiu and Bleecker 2001 a, b; Osakabe et al. 2013). Of the more than 610 genes that encode RLKs and RLPs in *Arabidopsis* genome, only a fraction has been assigned to the biological processes that they control (Di évart and Clark 2004).

The ERECTA family

The ERECTA family of LRR-RLKs, consisting of ERECTA (ER), ERECTA-like1 (ERL1), and ERECTA-like2 (ERL2), exhibits partial redundancy among these three members and mediates cell fate specification during the development of the stomatal complex (Pillitteri and Torii 2012). A mutant of TOO MANY MOUTHS (TMM) was first isolated as a phenotype that has stomates formed in adjacent cells that in wild type would have been developed into epidermal pavement cells (Shpak et al. 2005). The *tmm* plants exhibit an organ-dependent phenotype, with clustered stomata in cotyledon and leaves, whereas hypocotyls and stem are devoid of stomata. TMM thus can either influence stomata initiation in a positive or negative fashion (Geisler et al. 1998; Yang and Sack 1995). Since TMM encodes an LRR-RLP lacking any cytoplasmic effector domain by itself, it exerts its' effect by associating with ERECTA family receptors as co-receptor to perceive their putative ligands, EPFs/EPFLs (Pillitteri and Torii 2012).

The Epidermal Patterning Factor (EPF), EPF1 and EPF2, are three cysteine-rich peptides (CRPs) that mediate divers aspects of cell-cell communication (Marshall et al. 2011). ER is the main receptor for EPF2, together forming a ligand-pair *in vivo*, governing the initial decision of protodermal cells to generate a stomatal complex by asymmetric division. The EPF1-ERL1 pair acts to maintain stomatal cell activity and suppress guard mother cell (GMC) differentiation (Hara et al. 2007; Lee et al. 2012; Ohki et al. 2011). The signals received by kinase receptors are transmitted to the downstream MAPK cascades consisting of YODA (MAPKKK), MKK4/5 (MAPKKs) and MPK3/6 (MAPKs) to inhibit phosphorylation of basic helix-loop-helix (bHLH) transcription factors, such as SPEECHLESS (SPCH), MUTE and FAMA, leading to a suppression of stomatal cell fate specification (Wang et al. 2007). Stresses such as low temperature, drought, wounding, pathogens and stress-related molecules and hormones can either activate MAPK cascades or its target bHLH transcription factors to control stomata development (Pillitteri and Torii 2012).

BRI1 kinase

Brassinosteroids (BRs) play crucial roles in various aspects of plant growth and development, including cell elongation, photomorphogenesis, xylem differentiation, and seed germination (Gonz ález-Garc á et al. 2011; Hacham et al. 2011; Howell et al. 2007). Brassinosteroid-insensitive1 (BRI1) has been identified as the plasma-membranes receptor of brassinolide, the most active brassinosteroid (Wang et al.

2001; Li and Chory 1997). In this hormone-regulation pathway, BR binding to the LRR-RLK BRI1 inactivates BIN2, a glycogen synthase kinase-3, through the activation of phosphatase BSU1. By dephosphorylating transcription factor BZR1 and BES1, BSU1 positively regulates BR signaling, while BIN2 negatively regulates BZR1 and BES1 by phosphorylating them (Tang et al. 2008). Although, both BRI1 ASSOCIATED KINASE1 (BAK1) and BR-signaling kinases (BSKs) interact with BRI1 *in vitro* and *in vivo*, they play very distinct roles in BR signaling. BAK1 encodes an LRR-RLK that mainly mediates activation of BRI1 kinase, which enhances signaling output though reciprocal BRI1 transphosphorylation (Li et al. 2002; Divi et al. 2010; Russinova et al. 2004; Fàbregas et al. 2013). In contrast, BSKs directly mediate signal transduction from BRI1 to downstream BR responses (Tang et al. 2008).

Another feature for BR is their potential to enhance tolerance in plants to a range of abiotic stresses such as low/high temperature, drought stress, salt stress, and pathogen attack (Krishna 2003; Elhiti et al. 2013). The molecular mechanism of BR-induced stress tolerance is still largely unexplored (Elhiti et al. 2013). Recently, Kim et al. (2012) reported that BR inhibits stomatal development by alleviating GSK3-mediated inhibition of the MAPK module, leading to a decrease in stomatal density, that effectively limits water loss under high salt stress conditions (Ryu and Cho 2015). Since BR interacts with other hormones, some molecular changes associated with BR-induced stress tolerance result from BR cross talk with other hormones (Krishna 2003). For instance, BR positively regulates the salicylic acid pathway component NPR1 to promote thermo-tolerance (Divi et al. 2010; Ahammed et al. 2016), as well as WRKY70, which plays a pivotal role in salicylate-dependent and jasmonate-dependent defense pathways (Li et al. 2004, 2006). Another example of BR-other hormone cross talk is BR-enhanced salt tolerance which is established by either an ethylene-dependent or an ethylene-independent pathway (Divi et al. 2010; Ryu and Cho 2015). However, whether BRI1 participates in these stress responses signaling is unknown, further studies need to be done in the future.

FLS2

In *Arabidopsis*, the leucine-rich receptor kinase FLAGELLIN-SENSITIVE2 (FLS2) is involved in the recognition of flagellin, a protein that is part of the bacterial flagella, as a signal of bacterial presence, binding of which leads to the activation of defense responses (Gómez-Gómez and Boller 2000; Chinchilla et al. 2006). The *fls2* mutant allele *fls2-24* and *fls2-17*, with point mutations in the LRR motif of the extracellular domain and kinase domain, respectively, were shown to confer insensitivity to flg22. However, flg22 binding was restored in the transgenic *fls2* plants expressing the wild-type FLS22 gene, indicating that both extracellular and cytoplasmic domains of FLS2 protein are required for flagellin binding (Gómez-Gómez et al. 2001; Chinchilla et al. 2006). Another RLK, BAK1 was shown to interact with FLS2 *in vivo*, in a ligand-specific manner, suggesting a role for BAK1 in innate immunity of plants (Chinchilla et al. 2007; Lin et al. 2014; Heese et al. 2007). BAK1 has been considered a co-receptor for BR-binding BRI1, thus FLS2

interacts with BAK1 which subsequently phosphorylates BIK1, a cytoplasmic kinase, to control FLS2 signaling as a positive regulator (Wang 2012; Kemmerling et al. 2007; Schwessinger et al. 2011). Albrecht et al. (2008) reported that brassinosteroids inhibit the FLS2-mediated immune signaling independent of the complex formation with BAK1. BRs were also shown to inhibit downstream signaling triggered by the BAK-independent recognition of the fungal PAMP chitin (Albrecht et al. 2012). However, Belkhadir et al. (2011) provided evidence that BR acts antagonistically or synergistically in the response to microbe-associated molecular pattern (MAMP) through both BAK1-dependent and -independent mechanisms. It thus seems that the relative levels of BR, BRI1 and BAK1 determine whether BAK1 has a positive or a negative effect on FLS2-mediated signaling and that appropriate levels of endogenous BR are required for optimal *flg22* signaling.

RPK family

RECEPTOR-LIKE PROTEIN KINASE1 (RPK1) is an ABA-inducible LRR-RLK isolated from *Arabidopsis thaliana* and is expressed ubiquitously in flower, stem, leaves and roots. ABA is the plant hormone that is mainly associated with its role in stress signaling (Malamy 2005). RPK1 expression is rapidly induced upon treatment with ABA and by several environmental stresses, such as low temperature, high salt and dehydration (Smith and De Smet 2012; Lucas et al. 2013), indicating that the gene is involved in a general stress response. Loss of function of RPK1 resulted in ABA insensitivity and decreased expression level of ABA-responsive genes indicating that RPK1 functions as a positive regulator of ABA signal transduction (Osakabe et al. 2005).

Reactive oxygen species (ROS) are sub-products of aerobic metabolism in plants and other aerobic organisms (Apel and Hirt 2004). Various abiotic stresses lead to the accumulation of ROS in plants and an elaborate plant ROS network, comprised of efficient enzymatic and non-enzymatic antioxidant defense systems, is present to maintain the ROS level low, to protect plant cells from oxidant damage (Gill and Tuteja 2010; Gechev et al. 2006). ROS could also serve as signaling molecules to modulate various processes, including plant stress responses, program cell death, and stomatal behavior (Adler et al. 1999; Suzuki and Mittler 2006; Apel and Hirt 2004; Gechev et al. 2006). In a microarray analysis, ROS genes were identified in the *RPK1* knockout mutants and antisense transgenic plants, as well as water stress-responsive genes, which were also identified and up-regulated in *Arabidopsis* RPK1-overexpressing plants (Osakabe et al. 2013; Osakabe et al. 2005). Therefore, RPK1 seems to controls ROS homeostasis and the related, ROS-mediated water-stress and oxidative response pathways in *Arabidopsis*.

RECEPTOR-LIKE PROTEIN KINASE2 (RPK2), also known as RPK1/ TOADSTOOL2 (RPK1/TOAD2), is an important regulator in plant development, controlling cell fate in anther development (Mizuno et al. 2007), regulating embryo development during early globular stage (Nodine et al. 2011), and controlling plant meristem maintenance through CLV3 signal independent of the two best known

pathways: the CLV-CLV3 homomers and CLV2-CRN/SOL2 heteromers (Kinoshita et al. 2010).

PERK family

The *Arabidopsis* proline-rich extension-like receptor kinase (PERK) family consists of 15 predicted receptor kinases, is related to the *Brassica napus* PERK1 and shares sequence similarity with plant cell WALL-ASSOCIATED KINASEs (WAKs) (Silva and Goring 2002; Nakhamchik et al. 2004; Osakabe et al. 2013). The *Brassica napus* Bn-PERK1 was first reported to be involved in the early phase of perception and response to wounding or exposure to pathogens (Silva and Goring 2002). Antisense down-regulation of Bn-PERK expression in *Arabidopsis* results in phenotypic changes, like loss of apical dominance, increased secondary branching and floral organ defects (Humphrey et al. 2007; Haffani et al. 2006). PERK4 was identified as a positive regulator in the ABA response and the *perk4* T-DNA insertion mutant plant shows decreased sensitivity to ABA for seedling growth and root tip growth. Both [Ca^{2+}] channel currents and the cytosolic free calcium concentration are lower in *perk4* root cells than in wild type cells in the presence of ABA (Bai et al. 2009). This implies that PERK4 functions in the early stage of the ABA signaling pathway to modulate root cell elongation via [Ca^{2+}], a second messenger that participates in many aspect of plant growth and development, as well as in the response of plants to biotic and abiotic stresses (Bothwell and Ng 2004; Harper et al. 2004; Hetherington and Brownlee 2004).

Other stress responsive RLKs

RLK7, belonging to the LRR-RLK XI subfamily, was identified to be involved in the control of the timing of seed germination and tolerance to oxidative stress (Pitorre et al. 2010). GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1) is a critical early component in ABA signaling and mediates ABA- and H_2O_2-regulated stomatal movement in response to drought stress (Hua et al. 2012). *Srlk*, a novel LRR-RLK gene forms the legume *Medicago truncatula*, is rapidly induced by salt stress, and Srlk was shown to control the expression level of several salt-responsive genes (de Lorenzo et al. 2009), suggesting that it is involved in the adaptation of *Medicago* roots to salt tolerance. CRK36, a cysteine-rich RLK, interacts with and phosphorylates ARCK1, a receptor-like cytosolic kinase gene induced by abiotic stress, to form a complex that functions in a negative feedback mechanism regulating ABA and osmotic stress responses (Tanaka et al. 2012; Wrzaczek et al. 2010). However, as the ligands and kinase functions for these RLKs have not been resolved, further studies are required to elucidate how they are involved in sensing external signals and control downstream signaling pathways in response to various stresses.

Plants have evolved complex processes to adapt to and tolerate environmental stresses. The large membrane-anchored RLK protein families recognize these extracellular signals at the cell surface and activate the downstream signaling

pathways. The genome-wide collection of *Arabidopsis* insertion mutants presents us with the opportunity to get insight into the biological role of RLKs in response to these different environmental stimuli (Wang et al. 2008; Alonso et al. 2003). Generally, RLKs perceive signals like hormones, small peptide or other molecules and physical stimuli to trigger the intracellular downstream events of RLKs, including the kinase MAPK, ROS signaling, cytosolic [Ca^{2+}] concentration, ABA signaling and metabolic adjustment, leading to an acclimation to the environment (Osakabe et al. 2013). Due to the functional redundancy between receptors on the one hand and combination of functions for a single receptor on the other, the roles of RLKs in plant development and defense responses appear complicated. Although, more than 610 RLK genes were identified in the *Arabidopsis thaliana* genome, only a fraction of them have been associated with biological functions yet, and even when the function is known, the upstream and/or downstream targets are often not clear (Afzal et al. 2008; Morris and Walker 2003). Mapping the intricate signaling web will allow us to better understand how plant cells communicate with each other and with their environments.

Aim and the outline of this thesis

Hidden from our view, root system is the first organ for plant sensing the adverse signals in soil. Growing roots on the surface of a semi-solid agar medium greatly facilitates the analysis of root system architecture under different abiotic stresses. To date, most studies on RSA have been performed on two-dimensional images of roots captured by digital cameras or scanners, and several sophisticated image analysis programs have been designed to increase the accuracy of measuring specific RSA traits, such as RootTrace (French et al. 2009), REGR analysis (Walter et al. 2002), KineRoot (Basu et al. 2007), and RootflowRT (van der Weele et al. 2003). These programs mainly focus on analyzing root growth from a time series of images, used for kinematic or morphometric analysis of root. The second class of the RSA program has been developed to quantify RSA traits across the entire root system, including WinRHIZO^{TM} (http://www.regentinstruments.com/assets/winrhizo_softwa-re.html), Delta-T-Scan (http://www.dianjianghk.com/v_1/272.aspx), WR-RIPL (http://www.rootimages.msu. edu), RMS (Ingram and Leers 2001), and EZ-Rizo (Armengaud et al. 2009).

Of the more than 610 members of the RLK family in *Arabidopsis*, only a fraction has been firmly given a function. The collection of gene-knockout mutants provides a direct route to determine the function of the gene product. *Agrobacterium* T-DNA tagging has been proven to be a very efficient method of identifying a wide range of gene-indexed loss-of-function mutants (Krysan et al. 1999; Feldmann 1993; Alonso et al. 2003). After the isolation of a homozygous mutant with only one T-DNA insertion present, the next step is to determine the consequences of the mutant gene on growth and development compared with wild type. The aim of this thesis is to determine the role of receptor-like kinases (RLKs) in the modifications of RSA induced by environmental changes and in response to various biotic and abiotic stresses. In the first chapter, we reviewed the structure and classification of plant RLKs, their known functions during root growth and development, and the role of RLKs in response to unfavorable stress conditions.

Chapter 2 provides an extensive description of the newly identified mutant of the LRR-RLK gene, *ROOT GROWTH IHIBITION RECEPTOR* (*RGIR1*), which displays a shorter primary root and less lateral roots when grown on agar medium under optimal growth condition in *Arabidopsis* seedlings. Seed size, seed germination, root phenotype and leaf phenotype of *rgir1* knockout mutant plants were measured to address a possible role for *RGIR1* gene during plant growth and development.

In chapter 3, a kinematic analysis of the growth of *rgir1* mutant and wild type seedlings was performed to identify the functions of RGIR1 on root growth. The root system architecture, root growth rate and root branching of mutant and wild type seedlings grown on medium supplied with salt, or grown in a low temperature growth chamber of *rgir1* mutants and wild type were analyzed. Our main aim was to test whether RGIR1 acts in the chill-tolerance or salt-tolerance pathways of plant.

Chapter 4 provides an overview of the common effects of growth medium ingredients on RSA of plants and the responses of root morphology of *rgir1* mutants on 1/2 MS medium with low pH and medium with salt or without sulfur.

Chapter 5 describes the effects of the agar composition on root skewing behavior and the interaction with salt and osmotic (high mannitol) stress. The impact of salt and mannitol on root skewing behavior of plant, including the growth phenotypes of root tip, the skewing direction, and the slanting angle of tip root deviation from the vertical axis are described.

Finally, based on the outcome of the described experiments in the thesis, chapter 6 presents a general discussion of abiotic stresses and the surface of the media on RSA of plant, and the role of LRR-RLK RGIR1 on root growth and development is debated under control and stress treatments.

Chapter2

RGIR1 is a leucine-rich repeat kinase that involved in root system architecture of *Arabidopsis thaliana*

Nana Yu, Marten Staal, J. Theo. M. Elzenga

Laboratory of Plant Ecophysiology, Groningen Institute for Evolutionary Life Sciences, University of Groningen, The Netherlands.

Abstract: Receptor-like kinases are localized in the plasma membrane of plant cells and have a wide range of functions during plant growth and development. Previously, we identified the *ROOT GROWTH INHIBITION RECEPTOR 1* (*RGIR1*), which encodes a LRR-RLK, displayed distinct root phenotype in the insertional mutagenized plants from *Arabidopsis* ecotype Columbia under standard growth condition. To identify the biological functions of *RGIR1*, two T-DNA insertional mutants (*rgir1-1* and *rgir1-2*), were used in a detailed screen for alteration of root system architecture under optimal growth condition. Whereas *rgir1-1* and *rgir1-2* have smaller seed size compared with wild type, no evidence was found of a direct link between RGIR1 with control of seed size. Low temperature (15℃) and high salinity (>100mM NaCl) delayed full germination from 1 to 5 days, but the final germination percentage was not affected when compared with standard growth conditions at 21℃. The only difference found between mutant and wild type during the whole germination process, was a higher germination percentage of *rgir1-2* on day 1 at high temperature (25℃). Seedlings of *rgir1-1* mutant showed a significantly reduced root length and root surface area compared to wild type ($P<0.05$) on agar plates, whereas no difference was found for *rgir1-2* mutant seedlings grown on the same plate. In addition, no difference was found in leaf phenotype both for *rgir1-1* and *rgir1-2* mutant plants. Taken together, our results indicate that RGIR1 only functions in root elongation and development of plant.

Introduction

Receptor-like kinases (RLKs) have been known to play major roles in integrating environmental signals in plants (Shiu and Bleecker 2001 a, b; Osakabe et al. 2013). After the first plant receptor kinase, *ZmPK1*, was reported in maize (Walker and Zhang 1990), more than 610 members of RLK genes have been identified in *Arabidopsis*, representing nearly 2.5% of all *Arabidopsis* protein coding genes (Shui and Bleecker 2001a). Like in animals, RLKs are generally assumed to be localized in the plasma membrane and can bind extracellular ligands. Based on the structure of the extracellular domain, leucine-rich repeat (LRR) receptor-like kinases, form one of the largest receptor gene families with more than 200 members, and are divided into 15 subfamilies (LRR I to LRR XV) in *Arabidopsis* (Shiu and Bleecker 2003; Gish and Clark 2011).

In *Arabidopsis*, some of the RLK genes are involved in the root apical maintenance (Wierzba and Tax 2013). Key factors that controlling the distal meristem during postembryonic root development including CLE40 peptide (Stahl et al. 2009), receptor kinase encoding gene ACR4 (Tanaka et al. 2002; Gifford et al. 2003), homeobox gene WOX5 (Sarkar et al. 2007) and transcription factors SCR/SHR (Helariutta et al. 2000; Laurenzio et al. 1996). ACR4, expressed in columella cells and columella stem cells, is positively regulated by CLE40 from mature columella cells, and the activated ACR4 up-regulates it's own expression and represses WOX5 expression, thereby restricting stem cell identity along the distal axis to columella stem cells. Although the organization of the *Arabidopsis* shoot meristem differs from the root meristem, and the regulatory genes for shoot and root stem cell described so far are different, these genes were confirm to play parallel roles in root and shoot apical meristem (RAM and SAM, respectively) maintenance. Moreover, CLV1, a key receptor-like kinase, which perceives CLV3 to restrict the expression of the homeodomain transcription factor WUS to the SAM (Clark et al. 1993; Clark et al. 1995), interacts with ACR4 to form a complex that binds CLE40 and reinforces the repression of WOX5 (Stahl et al. 2013). In addition to this CLE peptide/receptor like kinase pathway, BRI1 expressed in the epidermis and SERKs expressed throughout the root, promote WOX5 expression and cell cycle progression via SCR and SHR, respectively (González-García et al. 2011; Petricka et al. 2012; Du et al. 2012).

Lateral roots (LRs) of *Arabidopsis*, are derived from the pericycle cells within the differentiation zone of the main root and contain their own apical meristem when mature, through a series of seven distinguishable stages (Malamy and Benfey 1997; De Smet 2012; De Smet et al. 2015). However, not all initiated lateral root primordias (LRPs) can mature into a LR and emerge through the endodermis and cortical cell layers of the mature root (Malamy 2005). One intrinsic pathway for LRs development is controlled by the auxin-dependent induction of cell-wall-remodeling (CWR) genes, which promote cell separation before LRP developing (Swarup et al. 2008; Péret et al. 2009). In endodermal cells, auxin derived from the LRP triggers the expression of CWR enzymes by targeting auxin-dependent degradation of the

IAA/SHY2 repressor to initiate cell separation in this tissue. An auxin influx carrier, LAX3, within the cortex is induced after the degradation of the IAA/SLR repressor. Influx and accumulation of auxin causes up-regulation of CWR enzymes to initiate cortex cell separation and induces the expression of LAX3 in the epidermal cells, resulting the separation of epidermal cells.

Furthermore, the related LRR-RLKs HAE and HLS2 and their peptide signal IDA have roles in cell separation in this auxin-regulation pathway during LR development (Kumpf et al. 2013). IDA expression is induced by auxin, derived from the young LRP, and then binds to it's receptor in the endodermal cells, to trigger expression of CWR genes. Expression of IDA within the cortex and epidermal cells overlaying LRP is dependent on key regulators of the LAX3 and ARF7, which increase the expression IDA and the receptor genes, to trigger the expression of CWR genes that dissolve cell wall of cortex and epidermal cells and ultimately leading to the emergency of the lateral root emergency. Moreover, initially characterization and BL treatment of mutants suggest that TMK family of LRR-RLK and BRI1 are likely playing a role in the LR development (Dai et al. 2013; Kim et al. 2006; Bao et al. 2004; Yoshimitsu et al. 2011). However, detailed analysis need to be done to help determine their specific function in this progress.

Root hairs are approximately cylindrical extension of epidermal cells that important for plant anchoring to soil and nutrients acquisition (Grierson et al. 2014). Like many other members of the Brassicaceae family, the root epidermis of *Arabidopsis* possesses a distinct position-dependent pattern of root hair cells and non-hair cells (Dolan et al. 1994; Galway et al. 1994). In the N position, the WER and MYB23 proteins form an active complex together with TTG/GL3/EGL3 proteins, and the complex induces the expression of GL2 and non-hair cell differentiation genes, leading to non-hair cell type of epidermis cells (Galway et al. 1994; Bernhardt et al. 2003; Lee and Schiefelbein 1999). However, a high level of CPC is present in the immature epidermal cells in the H position, which forms an inactive complex with TTG/GL3/EGL3 proteins instead of WER, leading to the repression of GL2 and hair cell differentiation (Wada et al. 1997; Grierson et al. 2014). It is noteworthy that SCM, an LRR-RLK receptor, differs from these preceding genes, is proposed to enable immature epidermal cells to detect a positional signal and initiate differential accumulation of the WER and CPC regulators (Kwak et al. 2005). In addition, the FERONIA (FER) receptor-like kinase acts as upstream of the RAC/ROP-signaled pathways and controls the ROS-mediated root hair development at the initiation stage (Molendijk et al. 2001; Duan et al. 2010; Huang et al. 2013).

In an earlier study, 70 RLKs were identified in a proteomic analysis of plasma membrane vesicles using an optimized 2D-LC MS approach. Here, we report the isolation and characterization of one RLK gene At2g37050, named as *ROOT GROWTH INHIBITION RECEPTOR 1* (*RGIR1*), which encodes a putative leucine-rich repeat receptor-like kinase in *Arabidopsis*. Two T-DNA insertional mutant lines (*rgir1-1* and *rgir1-2*) were used to study the biological function of RGIR1 during

plant growth and development. Our results showed that low temperature and high salinity directly delayed the germination of plant seeds both for wild type and *rgir1* mutants. Whereas *rgir1-1* and *rgir1-2* have smaller seed size compared with wild type, no evidence was found of a direct link between RGIR1 with control of seed size in *Arabidopsis*. Moreover, roots of *rgir1-1*, but not *rgir1-2*, exhibit a reduced root length, suggesting that the mutation didn't affect the function of the kinase when inserted after the kinase domain. Despite the distinct root phenotype of *rgir1-1*, no difference was observed for cotyledon size on the agar and shoot phenotype in soil. Thus, these results demonstrated that RGIR1 is a growth regulator that mainly functions in processes involved in root development of plant.

Material and Methods

Isolation and sequence analysis of RGIR1

The gene with AGI code At2g37050 was identified in an earlier study on highly expressed LRR-RLKs in *Arabidopsis*, as having a mutant phenotype with shorter main roots and was named *ROOT GROWTH INHIBITION RECEPTOR 1* (*RGIR1*). The structural domains of LRR-RLK kinase encoded by *RGIR1* were annotated by using the SMART (Schultz et al. 2000) and Pfam (Sonnhammer et al. 1998) algorithms and databases. Predicted protein interactions were identified tentatively by consulting the STRING database (http://string-db.org/).

Plant material and growth conditions

The *rgir1-1* (Salk_143700c) and *rgir1-2* (Salk_071422c) mutant alleles are present in the T-DNA express Collection at Salk institute (http://signal.salk.edu/cgi-bin/tdnaexpress) and seeds of mutants were obtained from the Nottingham *Arabidopsis* Stock Center (NASC, http://arabidopsis.info/). Homozygosity of the T-DNA insertion for each allele was determined by a three-primer PCR method designed by using the SIGnAl T-DNA Verification Primer Design Tool (http://signal.salk.edu/tdnaprimers.2.html). The sequences of primers were: LP (5'-TTGGACCCGTAAAAGAATTCC-3') and RP (5'-GATAAATTTCGGGGC-TGAAAG-3') for *rgir1-1*, and LP (5'-CTTTTCTAATGGGGCCTCATC-3') and RP (5'-GAAAGCTTTGGTGTCAACTGC-3') for *rgir1-2*. They shared the same T-DNA left border primer LBb1.3: 5'-ATTTTGCCGATTTCGGAA-3', as also recommended by the SIGnAl Primer Design Tool.

Seeds of ecotype Columbia and the two mutant lines were sown in soil in a growth chamber with 16 hours light (around ~120 μmol m^{-2} s^{-1})/8 hours dark at 21 ℃ during day period and 18 ℃ during night at 72% humidity, and then transferred to a green house two weeks later. Leaf samples of 14 days old plants were used for verification of homozygous T-DNA insertion and seeds of mutant plants were harvested only when they were considered homozygous according to the result of the PCR reaction system as described above.

RT-PCR analysis

For semi-quantitative RT-PCR analysis, shoot and root tissue of 24-d-old *Arabidopsis* wild type plants (Col-0) and *rgir1* mutant plants, cultured on 1/2 MS medium with 1% sucrose, were harvested and frozen immediately in liquid nitrogen. Total RNA from the different tissues was extracted with NucleoSpin® RNA plant kit (Macherey-Nagel, Düren, Germany). cDNAs, which were synthesized from total RNA with superscript 2 reverse transcripts (Fermentas) and Oligo (dT) primer (Promega) in a total 20 µl reaction mixture, were PCR-amplified. For *rgir1-1* transcription detection we used the following primers: RGIR1-1F (5'-GGTCCTTAACTTACAGAATGAACC-3') and RGIR1-2R (5'-CCATCAAGCCATAACTCAACC-3'). For *rgir1-2* transcript detection we used the specific primers: RGIR1-2F (5'-GTGTCAACTGCCGGAACATA-3') and RGIR1-2R (5'-GAGCTGTTGGCTGCAATACT-3'). The RT-PCR amplified reaction were performed using the following program: 94 ℃ for 5 min, followed by 30 circles consisting of 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 90 s, followed by a 7 min incubation at 72 ℃. We followed the same protocol and conditions for the Actin2 gene with specific primers: Actin2-F (5'-GTTGGGATGAACCAGAAGGA-3') and Actin2-R (5'-GAACCACCGATCCAGACACT -3').

Seed size and seed germination assay

The Columbia ecotype (Col-0), which is the background genotype for both *rgir1* insertional mutant alleles, was used as control in this experiment. Seed size was measured using a commercial scanner as described previously (Herridge et al. 2011). Around five hundred seeds of wild type, *rgir1-1* and *rgir1-2* mutants were placed in a glass petri dish and then imaged using a flatbed scanner (Epson, Québec, Canada). The seed size was measured by the "particle analysis" macro of the image analysis program ImageJ version 1.47 (National Institute of Health, USA) and each experiment was repeated three times.

In order to avoid contamination by fungi and bacteria, seeds were gas-sterilized in a desiccator by exposing them for 3 h to the fumes of a solution of 100 ml bleach (4% NaClO) mixed with 5 ml of 37% HCl. Subsequently the seeds were transferred to agar plates, consisting of 1/2 MS medium, supplemented with 1% sucrose, 1% agar, 2.5 mM MES (Sigma) and set to pH 5.7 with KOH (0.1 mM). After stratification for 3 days in dark at 4 ℃, plates with seeds were transferred to growth chambers with temperature of 15 ℃, 21 ℃ and 25 ℃, respectively, to test the effect of temperature on seed germination. To test the effect of salinity on seed germination, seeds were transferred to the same 1/2 MS medium plates, but supplemented with 0 mM, 50 mM, 100 mM, 150 mM and 200 mM NaCl, respectively, in a growth chamber at 21 ℃ during day and night. All plates were placed vertically and all chambers had the same photoperiod of 16 hours light/8 hours dark with a light level of ~120 µmol m^{-2} s^{-1} and a humidity of 72%.

Characterization of RGIR1

All F1 seeds of wild type Columbia and *rgir1* mutant lines were harvested at the same time and were used to check root and leaf phenotype. In order to avoid infection by fungi and bacteria, seeds were gas-sterilized as described above, and then transferred to agar plates in petri dishes with 1/2 MS medium supplemented with 1% sucrose, 2.5 mM MES (Sigma), 1% agar and pH was set at 5.7 with KOH (0.1 mM). The seeds were vernalized at 4 ℃ for 3 days after sowing on agar plates, subsequently all petri dishes were transferred to a growth chamber at 21 ℃ under fluorescent light (16 hours light / 8 hours dark cycles, light level of~120 umol m^{-2} s^{-1} during day-time) with a 72% relative humidity.

Only seedlings that germinated at the same time were used for measuring the phenotypic parameters of roots and shoots. Twelve day-old seedling of wild type and *rgir1* mutants cultured on agar were imaged with a flatbed scanner and images were analyzed using the WinRHIZO software package (WinRHIZO 2009 a,b,c) connected to the scanner. After growing for 17 days on agar plates the seedlings were transferred to soil and moved to a greenhouse with natural light. After 27 days growth in soil, leaves of wild type and mutant plants were photographed (Cannon 550d, 17-225nm lens) and the leaf surface area was determined using the Analyze Particles module of the ImageJ software package.

EBL treatment

For epi-Brassinolide (EBL) treatment, wild type (Col-0), *rgir1-1*, and *rgir1-2* seeds were grown on the surface of solid media consisting of 1/2 MS basal salt medium, 2.5 mM MES, 1% agar, 1% sucrose, and supplied with EBL at concentrations of 1, 10 and 100 nM. The final concentration of 1 nM EBL was chosen to test sensitive response of *bri1-10* and *rgir1* mutants grown on 1/2 MS medium as described above. Seeds of Col-0, *rgir1-1* and *bri1-10* were directly germinated and grown on the surface of the media for 19 days and then seedling were transferred to pots with commercial potting soil (vermiculite) in the green house (16 hours light/8 hours dark) for another 56 days before cutting to analysis shoot and root phenotype. Only demi-water was used to water plants in pots in the green house.

Results

***RGIR1* encodes an LRR-I receptor like protein kinase**

The gene with AGI code At2G37050, here after termed *RGIR1*, contains 15 exons and 14 introns, and encodes a protein of 934 amino acids with a predicted molecular mass of 103.4 kD (**Figure 1 A**). To examine the function of *RGIR1*, two T-DNA insertion lines, *rgir1-1* (Salk_143700c) and *rgir1-2* (Salk_071422c), from the collections of T-DNA transformed *Arabidopsis* lines (ABRC), were characterized. Right insertion of T-DNA in the *RGIR1* gene gives a predicted and observed PCR product around 700-bp using right and border primers, while the size of product in the wild-type DNA is around 1100-bp with left and right primer (**Figure 1 B**). To

identify how the insertions affect the transcription of the mutated *RGIR1* gene, the transcription level of the gene in shoot and root were compared with wild type using RT-PCR. The expression of *RGIR1* was abolished both in *rgir1-1* shoot and root (**Figure 1 C**), whereas, the expression of *RGIR1* was only slightly decreased in the *rgir1-2* mutant plants shoot and root (**Figure 1 D**).

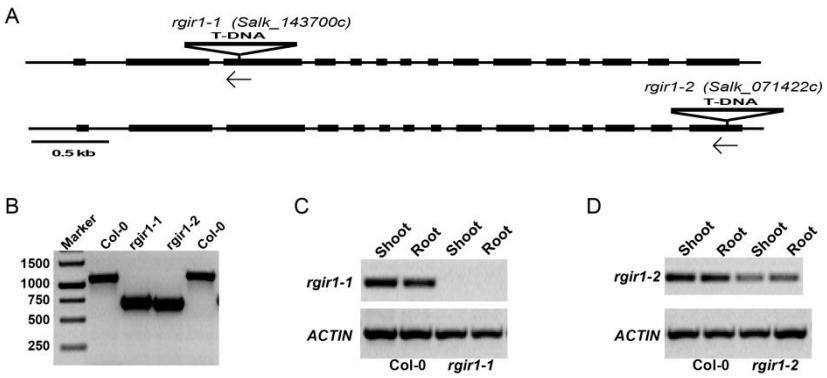

Figure 1. Identification of T-DNA insertions in *RGIR1* gene of *Arabidopsis thaliana*. A: Schematic showing the genomic structure of T-DNA insertion of two SALK lines of *RGIR1*, *Salk_143700c* (up) and *Salk_071422c* (down), named as *rgir1-1* and *rgir1-2* respectively. Exons are represented by black boxes, and black lines represent introns and 5' and 3' UTR. The triangle indicates the position where the T-DNA is inserted. **B:** Genomic PCR analysis of homozygous T-DNA insertion of *rgir1-1* and *rgir1-2*. By using a T-DNA primer and a gene specific primer, a PCR product around 700bp was amplified in mutant DNA but not from wild-type (Col-0) DNA, whereas, the PCR product with size around 1100-bp was amplified in wild-type (Col-0) DNA but not from the mutant DNA when using two gene specific primers spinning the insertion site. **C-D:** Transcription levels in homozygous mutants of *rgir1-1* (C), *rgir1-2* (D), and wild type. RT-PCR of total RNAs isolated from shoot and root of 24-d-old plants cultured on 1/2 MS medium. The Actin gene (At3g18780) was used as a control.

Sequence analysis of the RGIR1 protein revealed that it contains four distinct putative domains (**Figure 2**). The signal peptide domain, located at the N-terminus, consists of 23 amino acids, followed by a conserved Malectin-like domain from aa 31 to 365, as described for membrane-anchored endoplasmic proteins in animals (Schallus et al. 2008) and contributes to downy mildew disease (Hok et al. 2011). The P-kinase domain is located at the C-terminal of RGIR1 after the hydrophobic transmembrane domain of 23 amino acids, as found in most RLKs in plants. Comparison of database entries revealed that RGIR1 is a member of a subfamily of *Arabidopsis* LRR-RLKs (Shiu and Bleecker 2001). There are three conserved LRRs in the extracellular domain between the Malectin domain and the single transmembrane domain, suggesting that RGIR1 is a putative LRR-I transmembrane receptor kinase protein.

MVRISLLLLCLLVSTCLFTSSSA	23	signal peptide
QAPGFVSLDCGGAEPFTDELGLKWSPDNHLIYGETANISS	63	malectin like
VNETRTQYTTLRHFPADSRKYCYTLNVTSRNRYLIRATFL	103	
YGNFDNSNNVYPKFDISLGATHWATIVISETYIIETAELV	143	
FLASSPTVSVCLSNATTGQPFISTLELRQLSGSMYGSMLS	183	
EDRFYLSVAARINFGAESEASVRYPDDPYDRIWESDLQKK	223	
PNYLVDVAAGTVRVSTTLPIESRVDDRPPQKVMQTAVVGT	263	
NGSLTYRMNLDGFPGFGWAFTYFAEIEDLAEDESRKFRLV	303	
LPEQPEYSKSVVNIKENTQRPYRVYAPGYPNITLPFVLNF	343	
RFAKTADSSRGPILNAMEISKY	365	
LRKSDGSVDATVMANVASLYSSTEWA	391	3 LRRs
QEGGDPCSPSPWSWVQCNSDPQPR	415	
VVAIKLSSMNLTGNIPSDLVKLTG	439	
LVE**L**WL**L**DG**N**SFTGP**I**P**P**DFSRCPN	462	
LE**I**IH**L**EN**N**RLTGKI**P**SSLTKLPN	486	
LKE**L**YL**L**QN**N**VLTGTI**P**SDLAKDVI	510	
SNFSGNLNLEKSGDKGKKL	529	
GVIIGASVGAFVLLIATIISCIV	552	transmembrane
MCKSKKNNKLGKTSAELTNRPLPIQRVSSTLSEAHGDAAH	592	p-kinase
CFTLYEIEEATKKFEKRIGSGGFGIVYYGKTREGKEIAVK	632	
VLANNSYQGKREFANEVTLLSRIHHRNLVQFLGYCQEEGK	672	
NMLVYEFMHNGTLKEHLYGVVPRDRRISWIKRLEIAEDAA	712	
RGIEYLHTGCVPAIIHRDLKTSNILLDKHMRAKVSDFGLS	752	
KFAVDGTSHVSSIVRGTVGYLDPEYYISQQLTEKSDVYSF	792	
GVILLELMSGQEAISNESFGVNCRNIVQWAKMHIDNGDIR	832	
GIIDPALAEDDYSLQSMWKIAEKALLCVKPHGNMRPSMSE	872	
VQKDIQDAIRIEKEALAARGGISDEFSRSSAHSSSLNMGM	912	
LDLAGSQSYVSIDESVLQPTAR	934	

Figure 2. *RGIR1* encodes an LRR-I Receptor–like Protein Kinase. *RGIR1* encodes a polypeptide of 934 amino acids long and belongs to the LRR-I receptor-like kinase family of *Arabidopsis*. The extracellular domain of this protein contains a signal peptide of 23 amino acids, a Malectin-like structure, and 3 LRRs before the transmembrane region, followed by a P-kinase cytoplasmic kinase domain. The numbers on the right side of the figure indicate the position of all the amino acids residues, and the conserved residues of leucine-rich repeat are highlighted in bold.

Predicted functions of RGIR1

To identify novel receptors that may function together with RGIR1, a search of the *Arabidopsis* genome with the amino acid sequence of RGIR1 was conducted in the STRING database (http://string-db.org/). Four *Arabidopsis* genes were found that might be associated with *RGIR1*, including At5g08580 (EF-hand, calcium binding motif-containing protein), AT5G01150 (an uncharacterized protein), At2g28060 (5'-AMP-activated protein kinase beta-2 subunit protein), and AT2G23900 (Pectinlyase-like protein). However, no evidence has been shown that *RGIR1* has a direct interaction with these identified genes.

Seed size and seed germination

As shown in **Figure 3**, *rgir1-2* has the smallest seed size with ~0.13 mm^2 among the three genotypes. It showed a significant reduction of 13% compared to *rgir1-1* ($P<0.01$) and 15% compared to wild type ($P<0.001$). The germination rates were analyzed by scoring stage 0.5, characterized by radicle emergence (Boyes et al. 2001). At both the control (21 ℃) and high temperature (25 ℃), all seeds were germinated at day 2 after sowing, whereas at 15 ℃ the germination was delayed with one day (**Figure 4 A**). No differences were found between wild type and mutants in germination rate at 21 ℃ and 15 ℃. Strikingly, seeds of *rgir1-1* had the highest germination percentage at day 1 after sowing in the 25 ℃ chamber, with 10% higher germination than *rgir1-2* and 20% higher than wild type. However, the difference between mutant lines and wild type disappeared one day later, when all genotypes reached their final germination percentage.

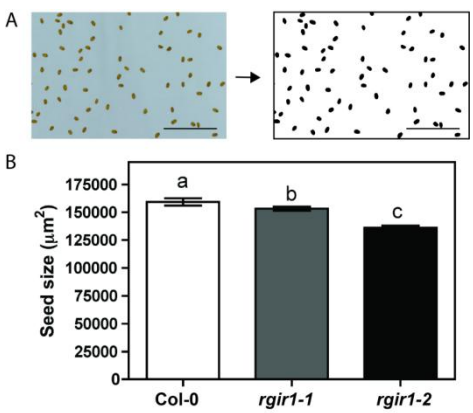

Figure 3. Seeds of *RGIR1* mutants are smaller than wild-type seeds. A: Images of seeds were taken by a scanner (Epson scanner) at a resolution of 1200 dpi using transmitted light (left), and the resulting image was processed to solid black and white using "threshold" function of ImageJ (right); Scale bar = 5 mm. **B:** Particle analysis of ImageJ was used to measure the seed size with a lower limit of 30000 um^2 to exclude any non-seed material. Different letters indicate significant difference between mutants and wild type (mean ± SD, $p<0.001$, Tukey's multiple comparison test).

High salinity (100 and 200 mM NaCl) delayed full germination by 1 and 5 days, respectively (**Figure 4 B**), whereas 50 mM NaCl had no effect compared with control condition. Germination percentages at 100 mM and 150 mM NaCl were similar (data not shown), with 70% at day 2 after sowing, followed by full germination at day 3. No significant differences were observed between wild type and the two mutants under the three salinity treatments. Surprisingly, nearly 100% of the seeds were germinated at day 7 after sowing, even at the high salinity concentration of 200 mM NaCl. At this treatment the two mutant lines seemed to have a higher germination rate than the wild type, however, this difference was not significant (**Figure 4 B**). Although seeds of wild type and both mutants all had a germination percentage of more than 95% at day 7 after sowing at 200 mM NaCl,

they stopped growth 3-7 days after germination at this high salinity treatment (data not shown).

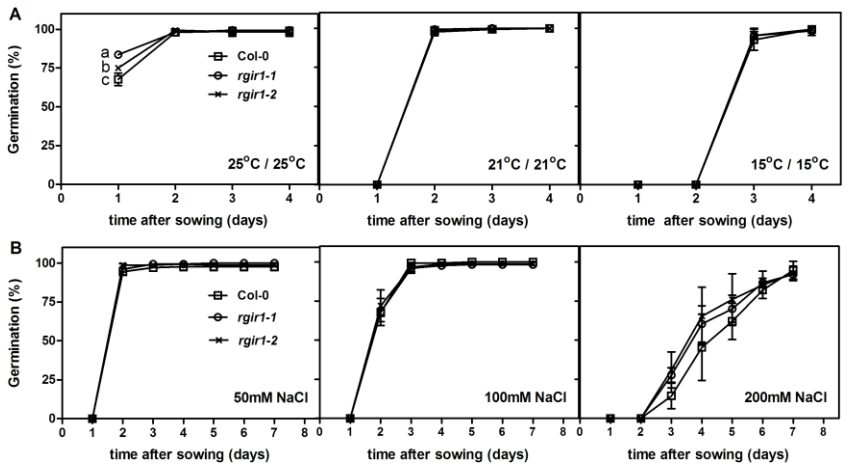

Figure 4. Seed germination time courses on 1/2 MS medium with 1% sucrose at different temperature (A) or on medium containing different concentrations salt at 21 ℃ (B). All growth chambers have the same light period of 16 hours light / 8 hours dark, and seed germination rate was scored every-day for three to seven days after vernalization in a cold chamber (4 ℃). Results are represented as the average value with standard deviation, n = 40. Different letters indicate significant difference between mutants and wild type (P<0.001, Two-way ANOVA).

Characterization of *rgir1* mutant lines

The main roots of 12-d-old seedlings of *rgir1-1* are significant shorter than wild type plants (P<0.05), and the main root surface area was also significantly smaller when compared to wild type (P<0.05) (**Figure 5**). However, seedlings of *rgir1-1* mutant had a similar root diameter and number of lateral roots as wild type plants. We did not observed any significant root phenotype change in the *rgir1-2* mutant plants compared to wild type plants, indicating that *rgir1-2* is a knock-down mutant and the insertion of T-DNA didn't affect its' function at protein level. When we transfer seedlings to soil after 27 days grown on MS medium, no significant difference was found between the mutants and wild type in leaf number and leaf surface area (**Figure 6**), suggesting that RGIR1 only functions in root growth and development of plants.

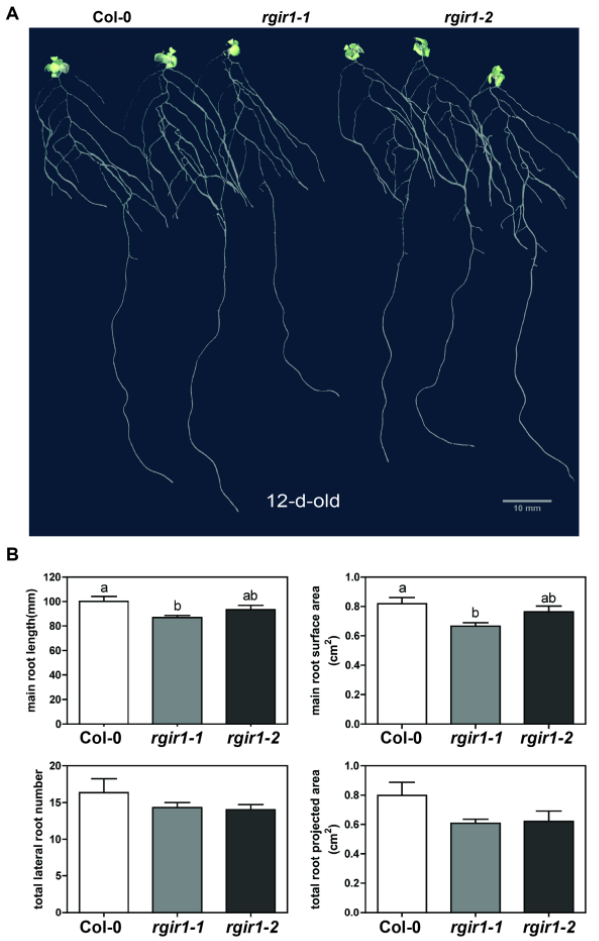

Figure 5. Quantitative analysis of root phenotype of *rgir1* mutant plants under standard growth conditions for *Arabidopsis.* A: Seeds of mutant and wild type were sown on 1/2 MS medium and plants of 12-d-old were scanned for quantitative analysis of root phenotype. Scale bar = 10 mm. **B:** Data of main root length, main root surface area, total lateral root number, and total root projected area were collected using root phenotyping program WinRHIZO^{TM}. Only lateral root length that longer than 0.5cm were selected for lateral root number calculation. Results are presented as mean ± SD, n=6. Different letters on top of bars represent significant difference between genotype ($P<0.05$, Tukey's multiple comparison test).

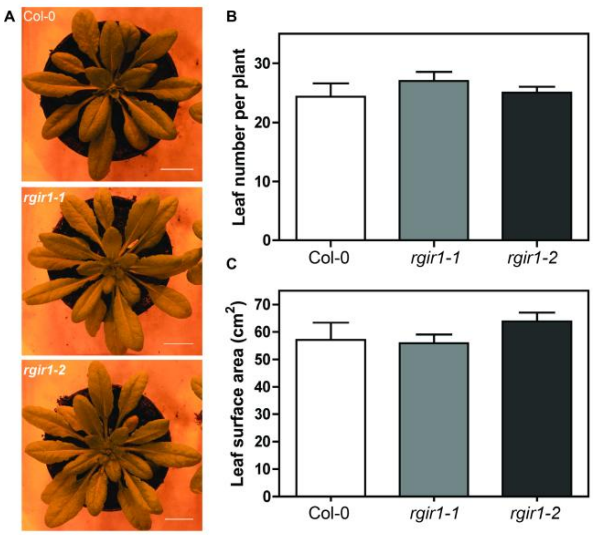

Figure 6. No difference was found between *rgir1* mutants and wild type for leaf phenotype when grown in green house. Plants of *rgir1* mutants and wild type were first cultured on 1/2 MS medium in a climate chamber at 21 ℃ with a photoperiod of 16 hours light/8 hours dark for 17 days, and then transferred to soil in a greenhouse with nature light. Images of leaf phenotype **(A)** were taken by a camera (Cannon 550d, 17-225 nm lens) 27 days later after transferred to soil, and leaf number **(B)** and leaf surface area **(C)** were calculated by Threshold function combined with Analyze Particles of ImageJ. Values represent mean ±SD, n=6. Scale bar = 2 cm in A.

BR effects on root growth of wild type and mutants plants

BSR050 (here named as *RGIR1*) was identified in a proteomic study of BSK3-interacting proteins in *Arabidopsis*, and the RNA expression of *RGIR1* in wild type seedling decreased slightly when grown on media with EBL (Xu et al. 2014). To test whether *RGIR1* encoded RLK is involved in BR responses, BR sensitivity of *rgir1-1* and *rgir1-2* were analyzed in a dose-response curve from 0 nM up to 100 nM EBL. Plates applied with EBL-free ethanol (95%) were used as reference. At 1 nM EBL, root growth of wild type was significantly increased, whereas, increasing EBL concentration significantly reduced main root growth of wild type when the concentration of EBL was higher than 10 nM (**Figure 7 A**). In response to EBL in the same concentration, root growth of *rgir1-2* was similar to that of wild type at all concentrations, but *rgir1-1* only showed similar root growth to that wild type and *rgir1-2* at 10 nM and 100 nM EBL (**Figure 7 A**). Although lower concentration EBL increased main root growth, roots of *rgir1-1* remained shorter than wild type on media with EBL less than 1 nM.

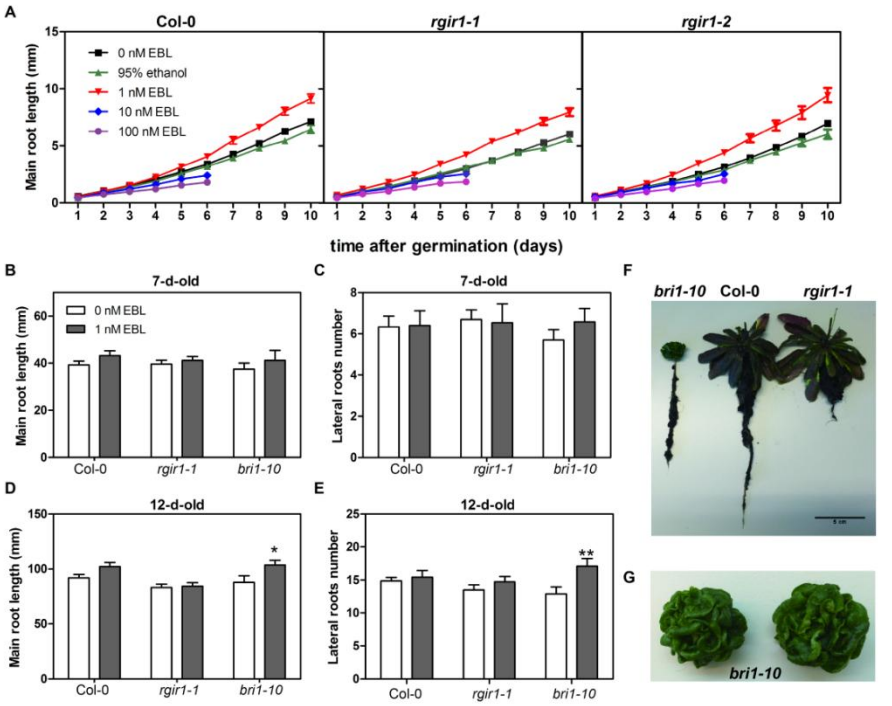

Figure 7. Effects of epi-brassinosteriod on root and shoot phenotype of Col-0, *bri1-10* and *rgir1* mutants. A: Main root growth analysis of wild type (Col-0) and *rgir1* mutant (*rgir1-1* and *rgir1-2*) seedlings grown on 1/2 MS medium containing different 24-epiBL concentrations and the blank solvent (96% ethanol). **B-C:** Main root length and lateral roots number of 7-d-old seedlings grown on medium with 1 nM or without 24-epiBL. Twelve seedlings were measured for each genotype. Error bars indicate SEM. **D-E:** Root length and lateral roots number of 12-d-old seedling grown on medium with 1 nM or without 24-epiBL. Twelve seedlings were measured for each genotype. Error bars indicate SEM. Two-way ANOVA analysis indicated the differences are statistically significant between treatments for *bri1-10* mutant seedlings. (*, P<0.05; **, P<0.01). **F:** Root system architecture of Col-0, *bri1-10*, and *rgir1-1* grown in vermiculite for 56 days after 19 days culturing on 1/2 MS medium with 2.5 mM MES, 1% sucrose, and 1% micro-agar. Scale bar is equivalent to 5 cm. **G:** Shoot phenotype of 76-d-old *bri1-10* mutant plants grown in a 22 ℃ growth room (16 hours light/ 8 hours dark).

Since BRI1 is a critical component of plasma-membrane receptor for plant brassinosteroids, *bri1-10* (Salk_041648), which causes BR-insensitivity dwarf and very reduced fertility (Kwon and Choe 2005), was introduced to test BR response together with *rgir1-1*. At 1 nM EBL, no difference was observed for main root length and lateral roots number of 7-d-old wild type seedlings grown on control medium (**Figure 7 B and C**). Compared with wild type plants, *rgir1-1* displayed indistinguishable root phenotype from wild type in the same conditions, and *bri1-10* showed shorter main root and less lateral roots but not significant statistically both on control and treatment media. At day12, the main root length of *rgir1-1* and *bri1-10* were significantly shorter than wild type on the control medium as well as the

number for lateral roots (**Figure 7 D and E**). Treatment with 1 nM EBL enhanced the main root growth and root branching both for 12-d-old wild type and mutant plants, but the promotion was much stronger in the *bri1-10* mutant plants.

The *rgir1-1* had shoot phenotype similar to that of wild type when they were grown in vermiculite for 56 days, but root system of *rgir1-1* was shorter and smaller than wild type in the identity condition (**Figure 7 F**). The *bri1-10* showed a significant dwarf shoot phenotype characterized by a dwarfed stature, dark green, thicken leaves, and very lower fertility (**Figure 7 G**). Although BR seemed to recover the root phenotype of *bri1-10* seedlings on the medium, root system of these two-month-old *bri1-10* seedling was shorter and smaller compared with wild type grown in vermiculites.

Discussion

There are more than 610 Receptor-like kinase genes in the *Arabidopsis* genome and these genes play important roles in various aspects of plant life (Shiu and Bleecker 2001). Over the years, an increasing number of characterized RLKs have been shown to play key roles in diverse and important biological processes, such as hormone perception, morphology development and change, pattern development, resistance to pathogens and abiotic stress tolerance (De Smet et al. 2009; Hazak and Hardtke 2016; Di évart and Clark 2004; Osakabe et al. 2013; Wierzba and Tax 2013). In this work, we present evidence that *RGIR1*, which encodes a putative leucine-rich receptor like kinase protein, plays critical role in *Arabidopsis* root development. Down-regulation of *RGIR1* transcription of T-DNA insertion in the promote region of *rgir1-1* resulted in a significant decrease of main root length and root surface area, whereas, the *rgir1-2* mutant plants showed similar main root length with ecotype Columbia (**Figure 5**). Sequence analysis showed that the T-DNA is inserted in the third exon of *RGIR1* gene in *rgir1-1* mutant plants, but it is only 24bp upstream of the end codon ATG in *rgir1-2* (**Figure 1**). Combined with the RT-PCR result (**Figure 1**), we found that *rgir1-2* is one knock-down mutant that has a somewhat lower expression of *RGIR1* both in shoot and root of the plants, but has a phenotype in both root and shoot similar to the wild type.

Reverse genetics has been used as an effective approach to illuminate biological functions of RLK genes of *Arabidopsis* (Alonso et al. 2003; Gou et al. 2010). In a search of genomic-wide transcriptome analysis, RGIR1 was found involved in the stress response of drought and freezing tolerance (Wituszyńska et al. 2013), in protein phosphorylation in response to various plant hormones and corresponded with quantitative trait loci that controls fiber length and lignin content of *Arabidopsis* stems (Capron et al. 2012). Moreover, comparison of the NORK/SYMRK region of legumes to other dicotyledonous plants showed that the closet homologues in *Arabidopsis* were the two RLKs At1g67720 and At2g37050 (here named as *RGIR1*) with a sequence identity of approximately of 33% (Stracke et al. 2002; Zhu et al. 2005; Kevei et al. 2005). Since SYMRK confirmed to function in early signal transduction between nod factor perception and activation of the legheamoglobin

genes, homologous RGIR1 RLKs might also involved in signal transduction of early root symbiotic.

Seed germination is controlled by environmental factors such as light, temperature, humidity and applied chemicals in the culture medium, as well as by genetic background. The size of the seed is determined by growth of both endosperm and integument, and some genes have been found that directly affect seed size of *Arabidopsis*, including IKU1 and IKU2 (Jofuku et al. 2005; Garcia et al. 2003), MINI3 (Luo et al. 2005), SHB1 (Zhou et al. 2009), ARF2 (Schruff et al. 2006), and TTG2 (Johnson et al. 2002). In this study, all genotypes had a germination percentage higher than 97%, both under standard temperature condition and higher/lower temperature, but the kinetics of the germination process was both affected by genotype and temperature conditions (**Figure 4**). Although T-DNA insertion mutant had smaller seed size than wild type before stratification, this did not delay germination when compared to wild type under standard growth condition and when exposed to salinity. Remarkably, the mutant *rgir1-1* showed a higher germination percentage at 25 ℃ at day1, indicating a possible role of gene *RGIR1* in seed germination process. Further studies are, however, needed to confirm a possible role of RGIR1 in the process of seed germination.

One of the best-characterized LRR-RLKs in *Arabidopsis* is BRI1, which perceives plant steroids hormone brassinosteroids at the cell surface (Li and Chory 1997), and numerous alleles of *bri1* were identified in a variety of independent screens (Clouse 2011). Recent evidences suggest that a large number of RLKs were confirmed to be involved in the BRI1 hormone signal transduction pathway and several members of BR-signaling kinases were phosphorylated by BRI1 before BR binding, which appear to play a redundant role in BR signaling (Tang et al. 2008). RGIR1, together with 11 transmembrane receptor-like kinases, was identified in a proteomics study of BR-signaling kinases3 (BSK3)-interacting proteins (Xu et al. 2014). In the BR-response experiment, the expression level of *RGIR1* decreased slightly by increasing concentration of EBL, indicating a role for RGIR1 in BR-regulated responses.

We found that BR is able to stimulate root elongation and growth in a dose-dependent manner (**Figure 7**). Compared with other tissues of *Arabidopsis*, the physiological response of different tissue varies in the concentration of exogenously supplied EBL (Müssig et al. 2003; Bao et al. 2004; Yang et al. 2005). In response to 1 nM EBL, both root length and lateral formation were significantly promoted for *bri1-10* root, while it was only slight but not significantly increased for wild type and *rgir1-1* plant. Thus, *bri1-10* might more sensitive to BR with shorter time treatment. Plants of *bri1-10* showed characteristic dwarf phenotype of BR-deficient and BR-insensitive mutants when grown in the soil and only watered demi-water, and the root system architecture was thinner and also decreased with shorter main root and less lateral roots, compared with wild type (**Figure 7**, Vogler et al. 2014). *rgir1-1* also displayed a smaller and compassed root system compared with wild type and *bri1-10*, and no difference was observed for shoot phenotype of plants grown in soil

(**Figure 6**), thus, the function of *RGIR1* mutation might mainly acts on the growth of main root.

Based on sequence similarity, BRL1 and BRL3 were identified possessing a ligand binding island domain similar with BRI1 and encode functional BR receptors to bind BL with high affinity (Caño-Delgado et al. 2004). Unlike BRI1 that is expressed in most cells of the plant, the expression of BRL1 and BRL3 are mainly in the vascular tissues, and they function redundantly with BRI1 in vascular development in the Col-0 background. Using IP and LC/MS/MS techniques, RGIR1 together with an ATP-binding cassette-2 transporter were found to co-immuno-precipitate with BRL3 in the provascular/stele tissues (Fàbregas et al. 2013), supporting the deduction that RGIR1 is involved in root growth and development. Taken together, our results indicate that LRR-RLK RGIR1 is involved in the root growth and development at later stage of plant growth both on agar plates and in soil. Although there is no direct evidence that RGIR1 is involved in the BR signal transduction pathway during root growth and development, some results seem to indicate such a role. The characterization of the *rgir1-1* mutant root phenotype provides a basis for further analysis of the role of gene *RGIR1* in plant growth and development.

Chapter3

Role of RGIR1 in controlling root system architecture of *A. thaliana*

Nana Yu, Jan Henk Venema, J. Theo M. Elzenga

Laboratory of Plant Ecophysiology, Groningen Institute for Evolutionary Life Sciences, University of Groningen, The Netherlands.

Abstract: The *ROOT GROWTH INHIBITION RECEPTOR 1* (*RGIR1*) gene encodes a receptor-like kinase that is involved in *Arabidopsis* root growth under optimal condition (Chapter2). In this study, kinematic and morphology parameters were measured to quantify the spatial distribution of growth rate and the cell numbers and size in the root tip of *rgir1* mutants and wild type under control conditions and when exposed to cold or salinity stress. Our results showed that the observed short root phenotype of *rgir1-1* mutant root was associated with a lower cell elongation rate and decreased cortex cell number in the transition and elongation zone of the root tip. In the presence of salt or cold stress, root growth and development in all genotypes (mutants and wild type) were strongly affected with shorter main root length and less lateral roots. The root phenotype of *rgir1-2* was similar with wild type Col-0 seedling under both control and stress (salinity and temperature) conditions. However, the *rgir1-1* seedling had shorter main root and less lateral roots than wild type under control condition. These differences between the rgir*1-1* and wild type were more pronounced at high temperature, but disappeared under cold and salinity stress. These results indicate that RGIR1 is a positive regulator of root growth in *Arabidopsis* under optimal growth condition and it seems not directly interact with pathways of plants in response to cold and salinity stress.

Introduction

Roots play vital roles during plant growth and development, including the uptake of water and nutrients, anchoring the plant into the soil, interacting with symbiotic fungi and bacteria, carbohydrates storage and influencing the rhizosphere by exudates, and have become more and more an indispensable part of the study on plant-environment interactions (Zhu et al. 2011; Sánchez-Calderón et al. 2013). Generally, the basic morphology of the root system is determined by inherent genetic factors, but abiotic (stress) conditions can strongly modify the root system architecture (RSA) through regulating primary root growth and lateral root initiation, and via formation of adventitious roots and root hairs (Osmont et al. 2007). The RSA is plastic and dynamic, allowing plants to respond to unfavorable environmental conditions, including nutrient deficiency, extreme temperatures, flooding, and salinity (Overvoorde et al. 2010; Galvan-Ampudia and Testerink 2011; González-García et al. 2011; Gruber et al. 2013; Nagel et al. 2009; Koevoets et al. 2016). However, the mechanism for the adaptation and alteration of the whole RSA of plant by single or multiple stress factors remains unclear.

Cold is a major abiotic stress that adversely affects plant growth and crop productivity (Yang et al. 2010). Low temperature not only decreases the elongation rate of primary root tips, but also affects the RSA by inhibiting the formation of lateral roots and the branching angle between primary and lateral roots (Nagel et al. 2009). The cold response of plants starts at the perception of the cold signal by membrane-located proteins, and this signal is relayed to downstream signalling components through a series of phosphorylation cascades resulting in an altered transcription of several cold responsive genes (Rahman 2013; Kazan 2013). Auxin plays major roles in the maintenance of cell division and expansion in the root apex, and strongly influences the initiation and development of lateral roots (reviewed by Nibau et al. 2008; Osmont et al. 2007; Péret et al. 2009; Petricka et al. 2012; Shibasaki et al. 2009). Low temperature inhibits both cell number and meristem size via the ARABIDOPSIS RESPONSE REGULATOR 1/12 (ARR1/12)-mediated reduction in the auxin accumulation in apex root, and cytokinin-signalling is also involved in the low temperature mediated inhibition of root growth (Zhu et al. 2015; Yang et al. 2017). Moreover, CYTOKININ RESPONSE FACTOR 2 (CRF2) and CRF3 are found to play an important role in regulating lateral root development in response to cold stress in *Arabidopsis* (Jeon et al. 2016).

High salinity also modulates primary root growth, development of lateral roots and/or root hairs, and the gravitropic growth of root tip (reviewed by Osmont et al. 2007; Galvan-Ampudia and Testerink 2011). Salinity stress is a combination of two different processes, both requiring plant adaptation. The first is an, often rapid, osmotic stress and the second is, a slower, ionic stress, caused by the toxic effect of high Na^+ concentration in the cytoplasm. Salinity tolerance mechanisms are a combination of osmotic tolerance and Na^+ and Cl^- exclusion, or Na^+ and Cl^- sequestration in, for instance, the vacuole (reviewed by Munns and Tester 2008). Many genes have been found to be involved in salinity tolerance mechanism, such as

SOS3 (with a role in sensing salinity in the root and transferring this signal to the shoot (Qiu et al. 2003; Ishitani et al. 2000; Shi et al. 2000), HKT (that acts as Na^+ transporter) (Rus et al. 2001; Rus et al. 2004; Mäser et al. 2002), and the vacuolar Na^+/H^+ exchanger AtNHX1 (that sequester Na^+ into the vacuole) (Møller and Tester 2007). In addition, plant hormones, which act as endogenous regulators of plant development, function as central integrators in conferring tolerance to unfavorable abiotic stress conditions including salinity stress (Ryu and Cho 2015; Iyer-Pascuzzi et al. 2011; Colebrook et al. 2014; Kohli et al. 2013; Kazan 2015).

Recent studies suggest that receptor-like kinases play a major role in relaying external abiotic stress signals to changes in gene transcription. For instance, a plasma membrane anchored Calcium/CAM-regulated RLK (*CRLK1*) plays a role in bridging calcium/calmodulin signalling and cold signalling and acts as a positive regulator of cold-regulated genes expression (Yang et al. 2010). The Salt Overly Sensitive 2 (*SOS2*) gene encodes a functional serine/threonine-type protein kinase that is predicted to be required for salt tolerance (Liu et al. 2000). Furthermore, other RLKs have been implicated in controlling disease resistance and functioning in defence responses, such as FLS2 (Gómez-Gómez and Boller 2000; Sun et al. 2013), PBS1 (Swiderski and Innes 2001), and WAK-like genes (Wagner and Kohorn 2001). Although our understanding of the functions of RLKs in all aspects of plant growth and development has increased in recent years, only a small number of members of this big gene family have been established biological roles and not too many genetic mutants of these related are available up to now.

Based on results of previous study in Chapter2 (not published), the *RGIR1* gene is believed to play a pivotal role in the root system architecture of *Arabidopsis*, with shorter main root length under optimal growth condition. However, the mechanism of how this newly identified RLK gene affects root growth or whether it is involved in the tolerance of cold or salinity is not yet clear. In the present study, we firstly performed a detailed root growth study of *Arabidopsis* ecotype Col-0 and *rgir1* mutants (*rgir1-1* and *rgir1-2*) at low or optimal growth temperature (21 °C) at different concentrations of NaCl, quantifying the relative elongation rate in the primary root growth zone with the aid of RootflowRT software (van der Weele et al. 2003). Our results indicate that RGIR1 inhibits root growth by reducing cell division in the transition zone of the root tip at optimal growth condition. Cold and salinity adversely affect root growth and lateral root development in wild type plants and *rgir1-1* and *rgir1-2* alike, indicating that RGIR1 is not involved in the growth response to low temperature or salinity.

Materials and methods

Plant materials and growth conditions

All homozygous F1 seeds of wild type Col-0 and two T-DNA insertion mutant lines of At2G37050 (referred to as *rgir1-1* and *rgir1-2*) were surface sterilized with gaseous chlorine and sown on 1/2 MS medium agar plates as described in Chapter 2. After sowing, plates with seeds were incubated at 4 °C for three days and

subsequently transferred to growth chambers with a relative humidity of 70% and a photoperiod of 16 hours light/ 8 hours dark. The irradiance level of light was about 110 µmol m^{-2} s^{-1} during daytime for all chambers. Effects of temperature treatment on root growth and development were performed in three climate chambers at constant temperatures of 15, 21, and 25 ℃, while the effect of salinity were performed at 21 ℃. For effect of salinity stress on root growth and development, seeds were germinated directly on 1/2 MS medium amended with NaCl to final concentrations of 0, 50 and 100 mM.

The seedlings on agar filled petri dishes were scanned daily with a scanner (Epson, Québec, Canada) to collect data on root system architecture analysis with WinRIZHO software (2009 a, b, c). Since seeds germination time differs under different treatments, day 0 was defined by radical emergence longer than 2 mm (48 hours after sowing at 25 and 21 ℃, and 72 hours at 15 ℃, whereas for salinity treatments this was 48 hours for plates with 0 and 50 mM NaCl, and 72 hours for the plates with 100 mM). Statistical analysis of data on main root length and lateral root density was done with the Prism Graphpad software package (Version 5, GraphPad Prism Software; San Diego, USA).

Kinematic analysis

To determine the effect of the *RGIR1* mutation and salt stress (50 and 100 mM NaCl) on cell expansion and division, we used a kinematic approach using the RootflowRT software package (version 2.8, University of Missouri-Columbia), which identifies the rate of expansion for different zones of apical part of the root (van der Weele et al. 2003). Apart from the velocity of the different root zones, the position of the first epidermal cell with a visible root bulge was determined. Six seedlings of both mutant lines and the wild type were grown on a vertically placed petri dish and images of the root tip under different culture conditions were taken with an Optikam B5 digital camera (Ponteranica, Italy) that was installed on an optical microscope (CX41, Olympus, Tokyo, Japan) using the 4X objective. For tip tracking, the tip was placed in the central field of view and images were taken every 20 s for a total length of time of up to 20 min. Nine images of each root tip were selected for calculation of spatial velocity using RootflowRT software with the time interval of 60 s. Only data with a reliable intermediate graph (automatically generated by the software) were used for further statistical analysis.

The velocity profiles were fitted with a logistic function with following model:

$$Y=S+ (L-S) / (1+\exp (-K*(X-X_0)))$$

in which Y is the predicted value, X is the distance to the quiescent center, X_0 is the midpoint of X value, K is the steepness of the curve, S and L are the minimum and maximum value of X at which breakpoint in the regression curve. The relative elongation rate was calculated by taking the derivative of the root growth velocity in relation to the distance from the root tip.

Salt growth responses

Experiments were conducted with seedlings of wild type Col-0, *rgir1-1*, and *rgir1-2*. Homozygous progenies of *rgir1-1* and *rgir1-2* seeds were surface sterilized as described above, and then sown on 1/2 MS medium together with Col-0 on the same plate. After stratification, plates with seeds were placed vertically in a climate chamber at 21 ℃. Three–day-old seedlings after germination were transferred to fresh medium that was supplemented with 0 (control), 50 or 100 mM NaCl.

Confocal microscopy

Root tips of 7-d-old seedlings grown on a 1/2 MS medium -/+ 50 mM NaCl were cut and stained with 10 μg/ml Propidium Iodide (PI) for 5 minutes at room temperature separately. Fluorescence signals were excited by a Kr/Ar 488-nm laser line using confocal laser-scanning microscope Leica TCS SP2 with a water immersion objective of 10X and the emission was passed through a 570-670 nm band-pass filter. The root meristem zone size and cortex cell numbers were calculated by visible cell borders that made by the intense PI fluorescence. Significance of the difference between wild type and mutants was tested by a one-way ANOVA followed by a Tukey's multiple comparison test.

Results

Temperature effect on root system architecture

At day12 after germination, low temperature (15 ℃) strongly affected root development and the resulting root architecture in *Arabidopsis* (**Figure 1 C**). However, the root system architecture both for mutants and wild type plant in the 25 ℃ chamber (**Figure 1 A**) were similar with those in the 21 ℃ chamber (**Figure 1 B**). The primary root length increased linearly for 12 days at the three temperature treatments (25, 21 or 15 ℃, **Figure 1 D-F**). Within 12 days, the total root length for the main root at 21 ℃ was not significantly different from 25 ℃, whereas, primary roots developed for 12 days at 15 ℃ were 40~60% shorter compared with roots developed at 25 ℃ and 21 ℃ (**Figure 1 D-F**). Differences between mutants and wild type roots became obvious 9 days after germination. At both 25 and 21 ℃, *rgir1-1* showed a significantly ($p<0.01$) shorter primary root length (**Figure 1 D, E**) at day 12 compared to the wild type, while *rgir1-2* showed a primary root length that was intermediate between (and not significantly different from) the wild type and *rgir1-1*. Moreover, the difference between *rgir1-1* and wild type became more exaggerated at 25 ℃ (**Figure 1 A, D**).

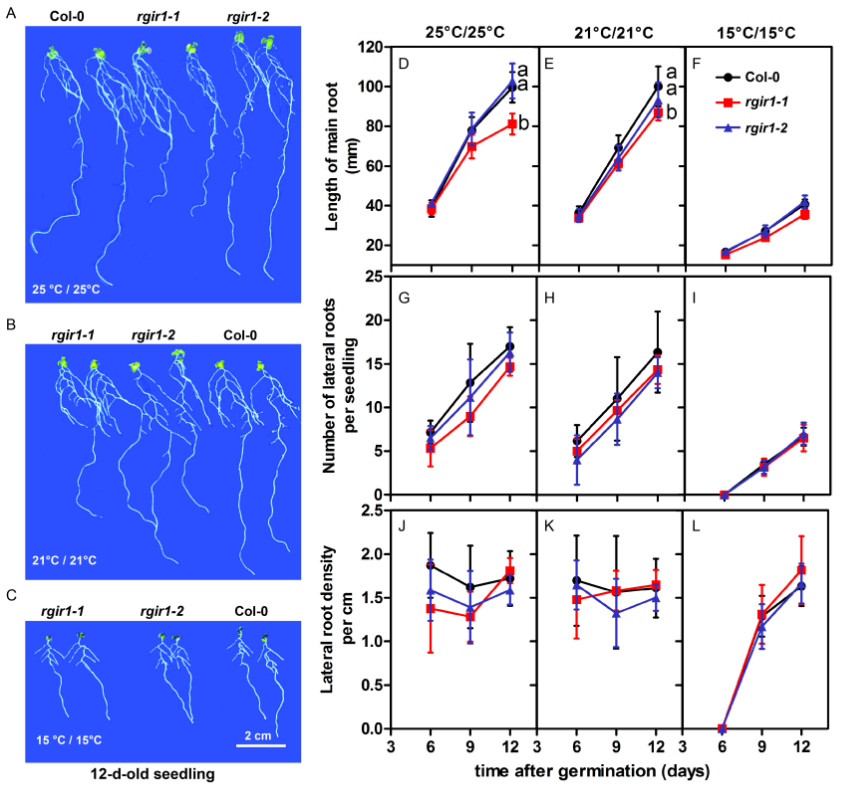

Figure 1. Effect of three constant temperature treatments on the root system architecture of two *rgir1* mutant lines and their wild type. A-C: Images of 12-d-old seedlings of *rgir1* mutants and wild type *Arabidopsis* ecotype Columbia grown on vertical 1/2 MS agar medium (with 1% sucrose) at 25 ℃ (A), 21 ℃ (B), and 15 ℃ (C). Scale bar = 2 cm. **D-F:** Main root growth of Col-0, *rgir1-1*, and *rgir1-2* roots grown at 25 ℃, 21 ℃, and 15 ℃. **G-I:** Lateral roots formation of Col-0, *rgir1-1*, and *rgir1-2* roots grown at 25 ℃, 21 ℃, and 15 ℃. **J-L:** The lateral root density was defined as the lateral root number per cm primary root, which was calculated by dividing the total lateral root number by the length of the primary root. Different letters depicted in D to F at day 12 indicate significant differences between *rgir1-1* and wild type as well as *rgir1-2* (means value ± SD, n=6, P<0.05, Two-way ANOVA).

The effect of temperature on lateral root development resembled the effect on primary root growth at day 12 after germination: total number of lateral roots significantly decreased (P<0.01) at 15 ℃ (**Figure 1 I**), compared with the standard growth temperature of 21 ℃ (**Figure 1 H**), while no difference was found between 21 and 25 ℃ (**Figure 1 G**). In contrast to the number of lateral roots formed, the lateral root density (number of laterals per cm primary root length) was not affected by temperature (**Figure 1 J-L**). Furthermore, no significant differences were found between wild type and mutants for both lateral root number and root density at any of the three temperatures.

Salinity effects on root system architecture

In accordance with the results of the temperature experiment, *rgir1-1* showed a significant shorter primary root length under control conditions (0 mM NaCl) at day 12 after germination, while no difference was found between the *rgir1-2* mutant line and wild type roots (**Figure 2 A and D**). At 50 mM NaCl, primary root growth was strongly inhibited by ~50% and no differences were found between mutants and wild type until day 12 (**Figure 2 B and E**). At 100 mM NaCl (**Figure 2 C and F**), primary root growth was severely reduced (80%) throughout the 12 days treatment when compared to conditions of 0 mM NaCl control treatment. Again, no differences could be observed between mutants and the wild type at 100 mM NaCl during the entire treatment period.

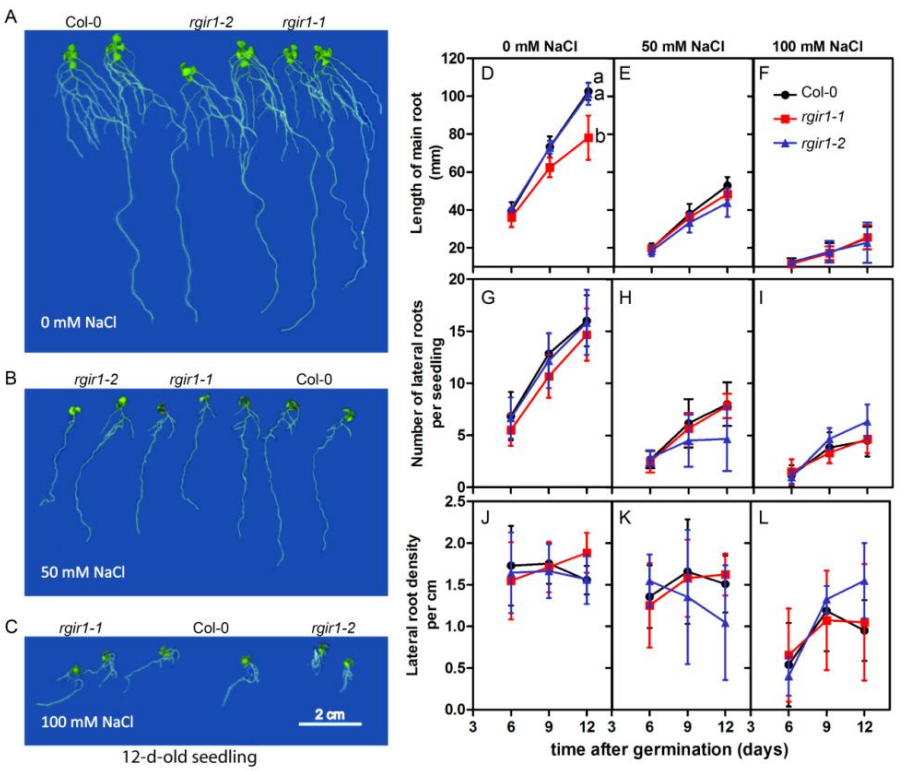

Figure 2. Effect of salinity stress on the root system architecture of two *rgir1* mutant lines and their wild type. A-C: Images for 12-d-old seedling of *RGIR1* mutants and wild type *Arabidopsis* ecotype Columbia grown on vertical 1/2 MS agar plates with 0 (A), 50 (B) or 100 mM (C) NaCl. Scale bar = 2 cm. **D-F:** Main root growth of Col-0, *rgir1-1*, and *rgir1-2* roots grown at 0, 50, and 100 mM NaCl treatment medium. **G-I:** Lateral roots formation of Col-0, *rgir1-1*, and *rgir1-2* roots grown at at 0, 50, and 100 mM NaCl treatment medium. **J-L:** Lateral root density was defined as lateral roots number per cm of each primary root, which was calculated by dividing the total lateral root number by the length of the primary root. Different letters depicted in D at day 12 indicate significant differences between *rgir1-1* and the wild type as well as *rgir1-2* (means value ± SD, n=6, $P<0.05$, Two-way ANOVA).

The total number of lateral roots without NaCl increased linearly during the 12 days treatment (**Figure 2 G**) and the lateral root density was between 1.5 to 2 per cm after initiation of lateral roots (**Figure 2 J**) both for mutant and wild type plants. Root branching was severely inhibited by the salinity treatment in a concentration dependent matter (**Figure 2 B, H, C, I**). No differences were observed between the two mutants and wild type in total lateral root number and lateral root density (**Figure 2 J-L**).

Response of *rgir1* mutants to salinity

To test the interaction between the *RGIR1* mutation and salinity stress, seeds of *rgir1-1* and *rgir1-2* were germinated on 1/2 MS medium, together with the wild type on the same plate to exclude possible systematic errors. Seedlings were transferred to new mediums with 0, 50 or 100 mM of NaCl after three days germination /development on the control medium without NaCl. As shown in **Figure 3 A**, 100 mM NaCl caused severe growth inhibition of the primary root and induced a curly root phenotype while seedlings at 0 and 50 mM NaCl were growing similarly and without phenotypically abnormalities. In our experiments the growth of *rgir1-2* and wild type were indistinguishable under all conditions. However, *rgir1-1* mutant displayed a significantly shorter primary root length ($p<0.05$) compared to wild type after 4 days grown on the new medium without salt. The difference became obscured between *rgir1-1* seedlings and wild type when grown on the medium in the presence of NaCl, and no difference was observed in root growth rate between mutants and wild type under different salt concentrations (**Figure 3 C**).

Velocity and strain rate profiles

The first epidermal cell with a visible root hair bulge is considered as a good parameter to quantify cell elongation under different treatments (Le et al. 2001). For a kinematic analysis of the root tip growth of *rgir1* mutants and wild type, the software package RootflowRT was used (van der Weele et al. 2003). The software calculates the rate of expansion in the different zones behind the root tip from high-resolution microscopic images taken with 20 s intervals. No visible root hair bulges were found within 1500 µm from the root tip of 6-d-old germinated seedlings of wild type and both mutants, whereas at day 14 root hairs already appeared at 1250 µm distance from the tip (**Figure 4 A and B**). The wild type did not differ significantly in the length between the root tip and the first visible root hair bulge at day 6 from the two mutants, but root hair bulges of 14-d-old seedling of *rgir1-1* emerged at a distance around 1200±125 µm from the quiescent center, which was significantly closer to the root tip than the root hair bulges of wild type and *rgir1-2* seedlings ($P< 0.001$, **Figure 4 B**).

The kinematic analysis confirmed that zone of the primary root that exhibits elongation growth at day 6 after germination encompassed the apical 1500 µm, while the zone was limited to the apical 1250 µm at day 14 (**Figure 4 C**). The profile of the elongation rate at day 6 peaks at about 800 µm from the apex, followed by a gradually decrease to zero at 1500 µm. At day 6 there is no difference between the

three genotypes in the position of the peak of the growth rate or the peak rate (between 35 and 40% h^{-1}). At day 14, the growth rate profile of *rgir1-2* and wild type were somewhat narrower, while the peak of maximum strain rate was not significantly shifted towards the root tip by about 100 µm compared to day 6. In contrast, the strain rate of *rgir1-1* at day 14 was strongly shifted apically to 500 µm from the tip and had a reduced lower maximum strain rate of only 22% h^{-1}, indicating that *RGIR1* affects root growth by reducing the elongation rate and not by narrowing the elongation zone.

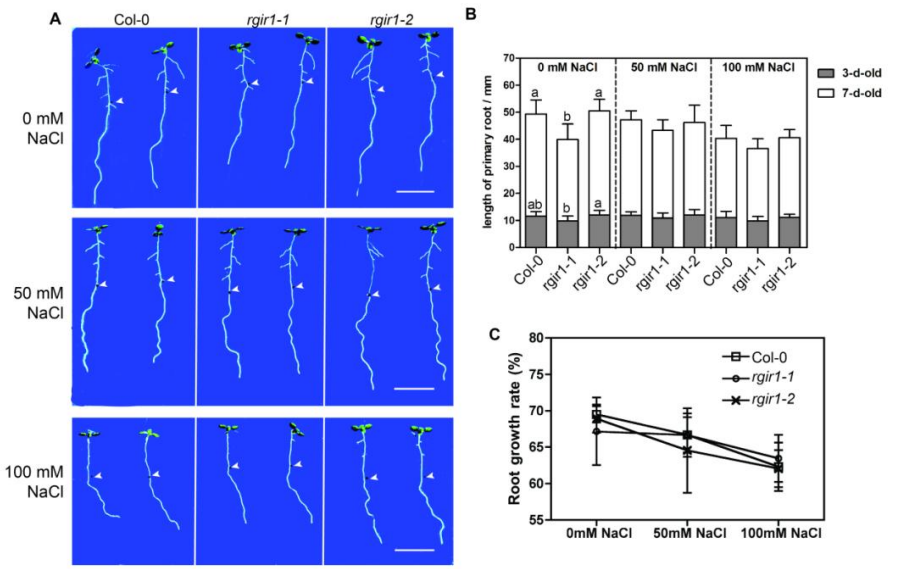

Figure 3. The response of root development of wild type and *rgir1* mutants plants to salinity. A: Seven-d-old roots of wild type (Col-0), *rgir1-1*, and *rgir1-2* plants at 0, 50 mM, 100 mM NaCl. Seeds were first germinated on a 1/2 MS medium without NaCl for 3 days and then transferred to media with different concentrations of NaCl. Arrows besides each root mark the position of root tip for 3-d-old plants at the time when transferred to the new medium. Scale bar = 10 mm. **B:** Length of the primary root for 3-d-old and 7-d-old plants shown in panel A (means ± SD, n=18). Different letters indicate significant differences between mutants ($p<0.05$, Two-way ANOVA). **C:** Dose response of plants shown in A to NaCl. Root growth rates at the three different NaCl concentrations (3 to 7 days after germination) are presented as a percentage of the root growth rates before transferring the roots from a medium without NaCl during the first 3 days after germination (means ± SD, n=18).

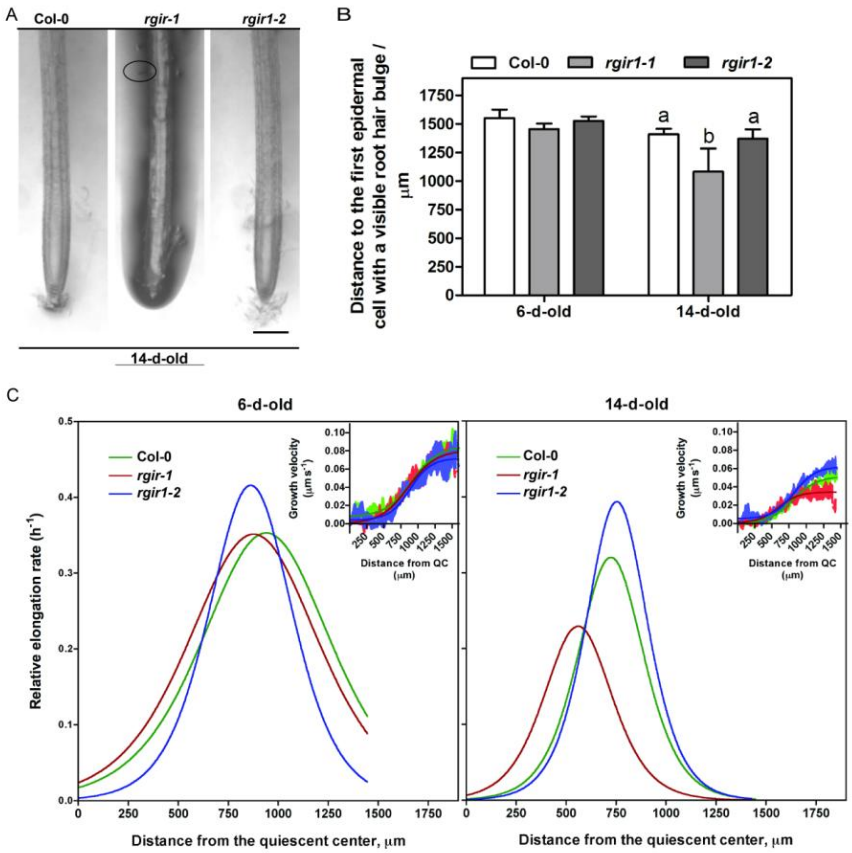

Figure 4. The *rgir1-1* mutant has a shorter meristem zone length compared to wild type (Col-0) and *rgir1-2* mutant line at later growth stage. A: Images of 14-d-old root tip with visible root hairs. The first visible root hair bugle is marked with the circle (scale bar = 2 cm). **B:** Distance to the first epidermal cell with a visible root hair bulge from the quiescent centre (QC) at day 14 (mean value ± SD, n=6). Different letters indicate statistically significant difference between genotypes (P<0.001, Two-way ANOVA). **C:** Spatial profiles of longitudinal growth velocity rate of 6 d and 14 d old *rgir1* mutant roots along the root growth zone. The insets show longitudinal displacement velocity profiles for the same roots (mean value ±SD, n=6).

In the presence of salt in the MS medium root hairs were formed closer to the tip of the root compared with control medium, in a concentration-dependent manner (**Figure 5 A**). The first visible bulge of an emerging root hair of wild type emerged at 750 μm from the QC in 100 mM NaCl, compared to 1500 μm in control medium or medium supplied with 50 mM NaCl (**Figure 5 B**). Root growth of wild type was severely prohibited under high salinity stress (**Figure 2**), thus the appearance of the first root hair under salt stress gives a good correlation with the main root length. While *rgir1-2* resembles wild type (**Figure 5 B**), *rgir1-1* showed a slight, non-significant, reduction in the distance between first root hair bulge and QC. Seedling of *rgir1-1* plants older than 9 d had significantly shorter main root than wild type and *rgir1-2* under control conditions (**Figure 2 A**). No difference was observed in 6-d-old between wild type and *rgir1-1* (**Figure 3 B**), but at 12-d-old a difference was obvious. Therefore, the lack of the correlation of root length with root hair formation in *rgir1-1* might be obscured by the lower growth rates and organ size in young plants.

Salinity does not affect the maximum cell elongation rate (around 30% ~ 40% h^{-1}) of wild type root, but the zone of maximal growth rate moved towards the QC by 200 μm and 300 μm under 50 mM and 100 mM NaCl treamtent, respectively, compared with control condition (**Figure 5 C**). Since root growth was seriously inhibited under high salinity stress, it seems that growth of main root doesn't correlate strongly with the distance between the QC and the zone of the maximum growth rate both for different genotypes and for salt treatment. Although *rgir1-1* had the lowest elongation rate compared with wild type and *rgir1-2*, the zone for elongation rate data and the zone for the maximum rate were not strongly affected under salt stress, indicating that salt doesn't have an additive inhibiting effect on the *RGIR1* mutation.

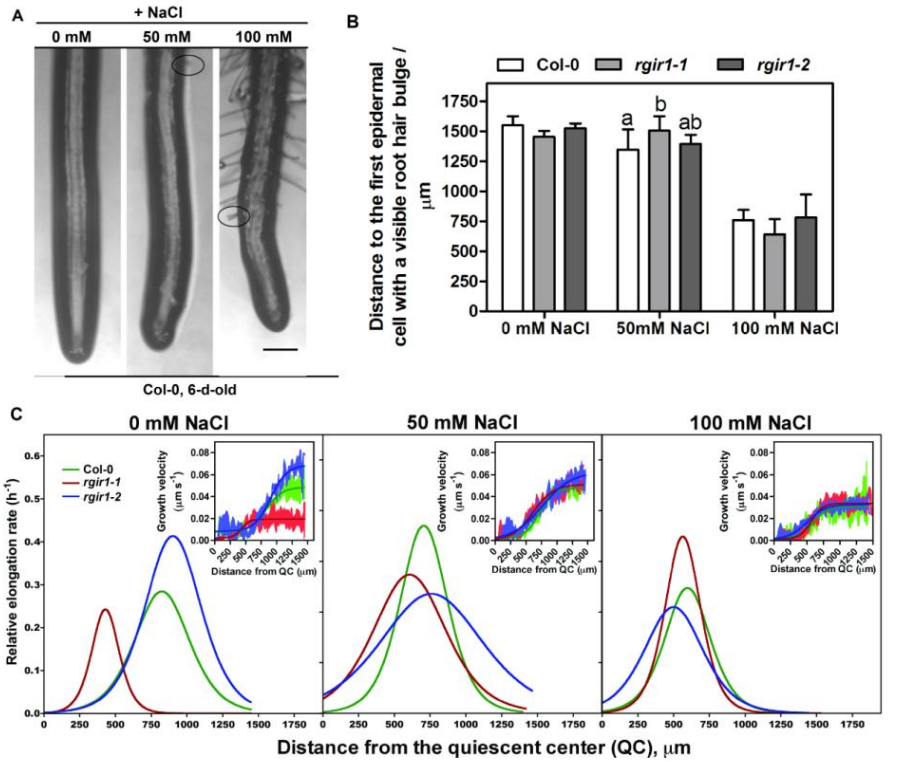

Figure 5. Root tip phenotype and relative elongation rate of *rgir1* mutants and wild type under salinity stress. A: Six-d-old root tip phenotype of wild type developed on 1/2 MS medium amended with 0, 50 or 100 mM NaCl. Circles mark the first visible root hair bulge. Scale bar = 200 µm. **B:** Distance from root tip to first epidermal root with a visible root hair of wild type and *rgir1* mutants treated on mediums with different NaCl concentrations (means ± SD, n=6). Different letters above bars represent statistically significant differences between genotypes ($p<0.05$, Two-way ANOVA). **C:** Relative elongation rate as calculated from the first derivatives of logistic growth curves fitted to the root growth velocity data (the growth data are represented in the insets). The growth data were obtained by tracking 500 points along the tip of root of mutants and wild type of 6-d-old seedlings (mean value ± SEM, n=6).

RGIR1 mutation controls cell division

To assess the role of the *RGIR1* mutation in root growth, we first compared the lengths of the apical meristem zone and the elongation zone of 7-d-old seedlings of mutant *rgir1-1* and wild type (Col-0). As shown in **Figure 6 A**, the apical root meristem zone is characterized by high rate of cell division from the QC to the first noticeable cortical cell, and the elongation zone of the root starts from the end of the apical meristem and extend to the start of differentiation zone with a visible hair bulge on the epidermal cell. The apical meristem length was reduced in *rgir1-1* compared with wild type ($P<0.001$) (**Figure 6 B**). The reduction in meristem size was even more pronounced in the *rgir1-1* mutant root when exposed to a growth

medium that was supplied with 50 mM NaCl (data not shown). The length of the elongation zone of *rgir1-1* is about 50% of that of the wild type (P<0.001, **Figure 6** C), and the corresponding cortex cell numbers showed a similar result, with a 10% decrease compared to wild type (P<0.05, **Figure 6 D**).

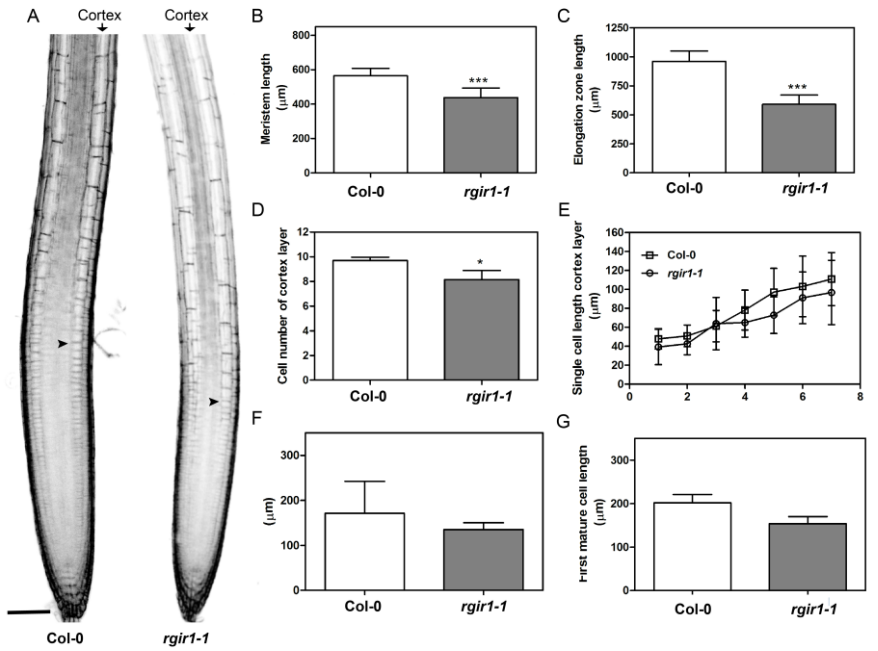

Figure 6. Root tip phenotype of Col-0 and *rgir1-1* mutant. A: Roots of 7-d-old seedlings grown vertically on 1/2 MS medium were stained with 10 μg ml^{-1} propidium iodide (PI) for 10 min at room temperature and visualized under Leica SP2 confocal microscope. Scale bar = 100 μm. Cortex layer was marked as the second layer from the outside (top) for wild type and *rgir1-1*. Black arrows indicate the first cortex cell with notable larger size. **B-C:** Direct measurement of the length of meristem and elongation zone. **D-E:** Cortex cell number in the elongation zone and the length of the first seven cells in this segment of root tip. **F-G:** Cell length for the first mature epidermal cell with a visible root hair bulge and the one before it in the elongation zone. Values represent the mean of ten plants for each mutant ± s.d. An asterisk indicates significant difference between *rgir1-1* and wild type (* = P<0.05; ** = P<0.01; *** = P<0.001).

We also studied the effect of *RGIR1* on the cell length in the cortex layer by direct measurement of all single cell lengths, from the first cortical cell that starts to elongate to the first epidermal cell with a visible root hair bulge. As shown in **Figure 6 E**, cell length increased gradually from the first cell to the 7th cell, which reached the maximum length about 120 μm to 140 μm. *rgir1-1* showed a slight but not significantly decrease in size at all cell positions. In addition, no difference was found between the mutant and wild type when we compared cell length for the last epidermal cell and first mature cell with a visible root hair bulge (**Figure 6 F and G**). These results show that *RGIR1* mutation impacts on root growth by controlling cell

division rather than cell expansion leading to less cell proliferation in the transition zone of the root.

Discussion

Root growth is determined by a balance of cell proliferation and cell expansion in the meristem and elongation zone of the root tip (Scheres et al. 2002). Cell numbers play an important role in regulating growth of plant organ especially for *Arabidopsis* root because of it's simple and stereotyped organization of cell types (Beemster and Baskin 1998; Dolan et al. 1993; Wildwater et al. 2005; Sarkar et al. 2007), and the first epidermal cell with a visible root hair bugle is generally accepted as the location along the root where cell elongation no longer takes place (Le et al. 2001). The kinematic results showed that inhibition of *rgir1-1* root growth is caused by a shortening of the division zone as indicated by the apical shift of the zone of maximal growth rate, rather than a reduction of the maximum expansion rate (**Figure 4**). According to the confocal results, the length of the cell division zone and the elongation zone are decreased in the *rgir1-1* mutant root while the number of cortex cell layers was less in *rgir1-1* than in wild type (**Figure 6**). However, the average length of cortex cells was similar as those wild type roots suggesting that *RGIR1* reduces root growth by affecting cell divisions in the transition zone rather than cell expansion in the elongation zone.

In roots of *Arabidopsis* seedlings, abiotic stress stimulates a stress–induced morphogenic response (SIMR), which is characterized by proliferation of lateral roots, inhibition of primary root and lateral root elongation, and alteration of cell differentiation status in the root apex (Zolla et al. 2010; Potters et al. 2007). Results from our study showed that both low temperature (15 ℃) and salinity, 50 mM NaCl, induced a change in the RSA of *Arabidopsis* characterized by a decrease in root length and less lateral roots. The effect of salt stress on RSA was even more pronounced under 100 mM salt treatment (**Figure 2 and 3**). When lateral root density was measured as number of laterals per cm primary root length, no difference was found between stress conditions at 15 ℃, nor at 50 mM NaCl treatment, compared with control condition at 21 ℃, suggesting there is a balance between growth and development in the intrinsic regulating pathways in response to diverse mild abiotic stresses.

As sessile organism, plants development a wide assortment of mechanisms to cope with the biotic and abiotic stress conditions in the surrounding environment (Pasternak et al. 2005; Munns and Tester 2008; Ryu and Cho 2015; Schmidt et al. 2015; Atkin and Tjoelker 2003) and the RLKs, localized in the membrane of plants, are important in the perception and transferring of signals during various aspects of root growth and in response to diverse stresses. *rgir1-1* mutant plants showed a reduced primary root length under standard growth conditions at 21 ℃, but do not exhibit a modified shoot phenotype (see Chapter 2, **Figure 6**). In the present experiments, a significant difference was found between *rgir1-1* mutant roots and its' wild type at high temperature treatment (25 ℃), where plants have a much higher

growth rate (**Figure 1 A**). However, in the cold- and salt-stressed *Arabidopsis* roots, there appeared to be no obviously correlation between the mutation of *RGIR1* gene with either cold (**Figure 1 C and F**) or salt stress (**Figure 2 and 3**) when root growth rates were lower and inhibited by cold /salt stress.

In the presence of salt (**Figure 3 and 5**), the decrease of the growth rate for *rgir1-1* mutant roots matched by a reduction of growth in wild type and *rgir1-2*, obscuring the phenotypic difference between *rgir1-2* and wild type. One possible explanation for the lack of difference in root growth under salinity stress between *rgir1-1* and wild type is that, the processes affected by salinity are no longer available for modification in the *rgir1-1* mutant background. Thus, the root growth phenotype for *rgir1-1* under control conditions is similar to those wild type plants under salinity and low-temperature conditions. When these *rgir1-1* roots are salt-stressed or developed in a cold chamber, the cell division zone for *rgir1-1* plants cannot be shortened any further by an additional abiotic stress factor.

In conclusion, our data suggest that this newly identified RLK RGIR1, is a positive growth regulator of root, and the short-root phenotype of the *rgir1-1* mutant is probably caused by reduction of the number of cortex cells in both the transition and elongation zone of the root tip, without affecting cell size. Under low temperature or salinity treatment, roots of *rgir1-1* mutant were phenotypically and physiologically similar as wild type, indicating that *RGIR1* gene-associated processes might partly overlap with the pathways involved in cold and salinity stress. Another possibility is that the effect of the *rgir1* mutant of abiotic stress leaves "no room for further inhibition" of growth by abiotic stress. Thus, root system architecture of *Arabidopsis* is controlled by a complex network and can be changed under changing conditions. Future research is needed to understand the molecular mechanism of RGIR1 and to find out what the specific role of RGIR1 in adaptation of root system architecture under abiotic/biotic stresses.

Chapter4

Effects of Growth conditions on root growth patterns in *Arabidopsis*

Nana Yu, J. Theo. M. Elzenga

Laboratory of Plant Ecophysiology, Groningen Institute for Evolutionary Life Sciences, University of Groningen, The Netherlands.

Abstract: Root system architecture of plants is regulated by both intrinsic and environmental response pathways. The goal for this study was to determine which of the components of the nutrient medium are having effects on root elongation or/and root branching, and how roots respond to abiotic stresses and nutrient deficiency. We found that sucrose induced a waving and skewing phenotype on hard agar, with strongly reduced lateral root formation and an increased length of the main. Although sucrose affected roots of wild type plants and *rgir1* mutants in a similar way, the *rgir1-1* mutant, which exhibits altered root growth kinetics in the transition zone, had a shorter main root. Inhibition of main root growth and root branching were observed on media supplemented with salt or mannitol, or with a low pH, while sulfur-deficiency did not affect main root length. In contrast, lateral root induction was strongly stimulated in media lacking sulfur. Roots of *rgir1* mutants reacted similar to wild type to salt and osmotic stress, but S-deficiency medium induced more lateral roots in *rgir1-1* mutants than in wild type plants, indicating a possible role for RGIR1 in the process of lateral root initiation or emergence.

Introduction

The *Arabidopsis* root has been used as a model for root growth and development, as it has a comparatively simple organization and can be easily cultured in non-soil media (Petricka et al. 2012; Osmont et al. 2007). Structure and composition of the growth medium can strongly modify root growth and architecture. When grown on the surface of nutrient containing agar medium, the combined effect of gravity and the contact between the medium and the root results in a characteristic skewing and waving growth pattern (Oliva and Dunand 2007; Johnsson et al. 2009; Scherer and Pietrzyk 2014), and this root surface-dependent behavior varies between different gel patches (Vaughn and Masson 2011; Thompson and Holbrook 2004). Also the pH of the root rhizosphere displays a heterogeneous patchiness when grown *in vitro* on agar plates, as well as in natural soils (Walter et al. 2009; Blossfeld and Gansert 2007; Hinsinger et al. 2003). Moreover, the low pH of the growth medium can severely inhibit root growth by decreasing the cytoplasmic pH of the root cells (Iuchi et al. 2007; Yan et al. 1992).

There is a large body of literatures describing how the growth medium can alter root system architecture in different ways (Malamy 2005). A major proportion for these papers is referred to morphogenetic responses of plants to salinity stress. This focus on salinity stress stems from the fact that this is one of the most significant factors limiting crop production and is a threat for future food supply for human (Kazama et al. 2013; Zhu et al. 2007; Boursiac et al. 2005; Møller and Tester 2007). Other growth medium factors are nutrients, such as nitrate, phosphate, sulfate and ion that act as environmental signals to trigger molecular mechanisms involved in the cell division and proliferation of plant roots (Lopez-Bucio et al. 2003; Vidal et al. 2008; Gruber et al. 2013), and hormones, which play vital roles in the intrinsic pathways that determine root system architecture (Muraro et al. 2014; González-García et al. 2011; Petricka et al. 2012; Overvoorde et al. 2010; Ryu and Cho 2015). In addition, osmotic stress caused by drought or high salinity in the medium also affects plant root system growth and development (Deak and Malamy 2005; Nguyen et al. 2016; Liu et al. 2014; Zwiewka et al. 2015; Osakabe et al. 2013).

Roots of *Arabidopsis* have developed a generic stress-induced morphogenetic response under distinct stresses on the grown medium. For instance, exposure to a mild salt stress causes a drastic reduction in main root and lateral root elongation, but an increase in lateral root number. Furthermore, this stimulation of lateral roots proliferation by salt stress is enhanced by nitrate in the medium (Zolla et al. 2010). Additionally, nutrient varieties and the nutrient availability in the agar medium also has a profound impact on root system architecture, by altering the length and diameter of main roots, inhibiting or stimulating lateral roots and roots hairs, and controlling root growth direction and branching angles of lateral roots (Singh et al. 2014; Booker et al. 2010; Malamy 2005; Gruber et al. 2013; Osmont et al. 2007). However, distinct effects on root system architecture strongly depend on kind and concentration of the nutrient involved (Gruber et al. 2013; López-Bucio et al. 2003; Hodge 2004). Despite the diversity in phenotype changing under different abiotic

stress conditions, the generic stress-induced morphogenic response comprise of inhibition of cell elongation, localized stimulation of cell proliferation, and alteration of cell differentiation status (Potters et al. 2007).

Lineages and fate of cells in developing *Arabidopsis* roots show a stereotypical pattern forming a set of concentric cylinders, consisting (starting from the outermost one) of the epidermis, the cortex, the endodermis and the stele (consisting of pericycle and vascular bundles), with the columella and lateral root cap providing additional layers at the root tip (Wierzba and Tax 2013; Scheres et al. 2002; Petricka et al. 2012; Yan et al. 1992). Each cell type in the root has its own transcriptional profile and many biological functions are regulated in a cell–type-specific manner (Birnbaum et al. 2003; Brady et al. 2007). Recently, comparison of gene expression levels within cell types under standard conditions with those under certain stresses, demonstrated that cell identity plays important roles in the stress responses of plants (Iyer-Pascuzzi et al. 2011; Dinneny et al. 2008). Thus, the use of environmental stimuli combined with the genomic-wide data sets allows the identification of associated regulator genes within cell types, and those root-patterning factors, which always are expressed at higher levels in the specific cell types regardless of the outside environment.

We previously identified a receptor-like kinase mutant, *rgir1-1*, which had a shorter main root and less lateral roots compared to the wild type *Arabidopsis* ecotype Columbia. In a screen for common stress response genes in the whole root, the gene expression of *RGIR1* was enriched in the stele and endodermis layer under low pH and sulfur deficiency stress conditions, respectively (Iyer-Pascuzzi et al. 2011). As detailed studies of wild type and *rgir1* mutant root responses under low pH and sulfur deficiency conditions are lacking, the specific role of RGIR1 in root development is still not elucidated.

In this present study, we set out to understand how nutrient and growth media affect root system architecture of *Arabidopsis* seedling grown on the surface agar plate. *Arabidopsis* ecotype Col-0 and two *RGIR1* alleles (*rgir1-1* and *rgir1-2*) in Col-0 background were used to investigate the response of root system architecture under different abiotic stress conditions. In particular, our objective is to identify the specific component of the growth media and to which extent that they affect root elongation and/or root branching. Our data showed that elongation of primary root was increased on the higher concentration agar while the average length of lateral roots was inhibited for the same plant. The length of primary root was increased in the presence of sucrose in the media, whereas, low pH and increasing salinity stress dramatically repressed root elongation and root branching of plants. No difference was observed between mutant seedlings and wild type seedling, when they had reduced root system under stress conditions. Root elongation was not affected on the sulfur deficiency media both for wild type and mutant seedlings. However, in *rgir1-1* roots starved with sulfate, lateral roots formation formed earlier and closer to the tip of the root compared to *rgir1-2* and wild type.

Materials and methods

Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0), *rgir1* mutants, *rgir1-1* (Salk_143700c) and *rgir1-2* (Salk_071422c) were used in this experiment. All wild type and mutant lines were in Columbia background and seeds were obtained from NASC (http://arabidopsis.info/). Seeds of mutants and wild-type (Col-0) were gas-sterilized in a desiccator for 3 hours with the fumes in a beaker with 100 ml of bleach (4% NaClO) mixed with 5 ml 37% Hydrochloric Acid and then sown on agar plates with nutrient media with different additives in 120×120 mm square Petri plate, approximately 2 cm from the top edge. Plates were wrapped with parafilm and, after two to four days vernalization at 4 ℃, transferred to a growth chamber with 16 hours light/ 8 hours dark at 21 ℃. All plates were placed vertically in the growth chamber, except when being scanned on a flatbed scanner for two to three minutes at the indicated days.

Media composition

The standard medium consisted of 1/2 MS (Murashige and Skoog 1962) basal medium, 2.5 mM MES, 1% agar, and adjusted to pH 5.7 with KOH (0.1 mM). In the experiments to test the effect of the agar concentration on root growth and development, the agar concentration was increased to 1.5% without any change for the other ingredients. Sucrose (1%) was added to the standard medium to test the effect of sucrose on root system architecture of plants grown on hard medium. For salt and mannitol treatment experiments, plants were directly germinated on hard (1.5%) standard medium with 1% sucrose and different concentrations of NaCl or mannitol, as indicated.

The sulfur sufficient media contained 1.25 mM KNO_3, 1.25 mM $Ca(NO_3)_2$, 0.25 mM KH_2PO_4, 0.5 mM $MgSO_4$, 22.5 μM Fe^{3+} (EDTA), 11.6 μM H_3BO_3, 2.4 μM $MnCl_2$, 0.24 μM $ZnSO_4$, 0.08 μM $CuSO_4$, and 0.13 μM Na_2MoO_4. To make S-free medium, $MgSO_4$, $ZnSO_4$, and $CuSO_4$ were replaced by their respective chloride salts. Low pH media is 1/2 MS salts with vitamins (Murashige and Skoog 1962), 2.5 mM MES, 1% agar, and pH adjusted to 4.6 by KOH (0.1 mM).

Root system architecture analysis on solid media

Seedlings of Col-0, *rgir1-1*, and *rgir1-2* that grown on different medias were scanned everyday by a scanner connected to computer running image analysis program WinRHIZO (van der Weele et al. 2003). The root architecture parameters, including the root length, cotyledon length, and lateral roots number, were determined. All data collected were statistically analyzed by a two-way ANOVA test using GraphPad prism (http://www.graphpad.com/).

Results

Gel concentration affects root waving but not root growth when placed vertically

To determine whether a higher concentration of agar in the medium affects the architecture of surface-grown root system, seedlings of *Arabidopsis* ecotype columbia and *rgir1* mutants in the Col-0 background were grown on 1% and 1.5% agar plates (without sucrose) and placed vertically or at 45°. When placed vertically, seedlings of 7-d-old wild type grew relatively straight regardless of the agar concentration, and no waves were observed near the tip of main root. Roots of *rgir1* mutants similarly displayed a straight main root without waves, both on control and hard medium when grown vertically. However, wild type and mutant roots skewed to the right (observed form the bottom of the plate) and started to make coils when the plates were placed at a 45° angle. The root phenotype of wild type and mutants grown on 1% medium, were indistinguishable from those grown on hard medium with 1.5% agar (data not shown).

We also compared root growth parameters between wild type and mutants on hard medium placed vertically. *rgir1-1* has significantly shorter main root length ($P<0.05$) compared with wild type both at 1% and 1.5% agar medium (identical to our previous results). The roots of *rgir1-2* were indistinguishable from wild type on 1% agar but significantly shorter ($P<0.001$) than wild type on vertical medium with 1.5% agar (**Figure 1 A**), indicating a possible role for harder agar concentration on the elongation of root. No differences between mutants and wild type both at control and hard medium were found in the number of lateral roots (**Figure 1 B**) and cotyledon length (**Figure 1 C**). However, *rgir1-2* showed the shortest length of lateral roots on 1% medium placed vertically, a difference that disappeared when grown on hard medium at 1.5% agar (**Figure 1 D**).

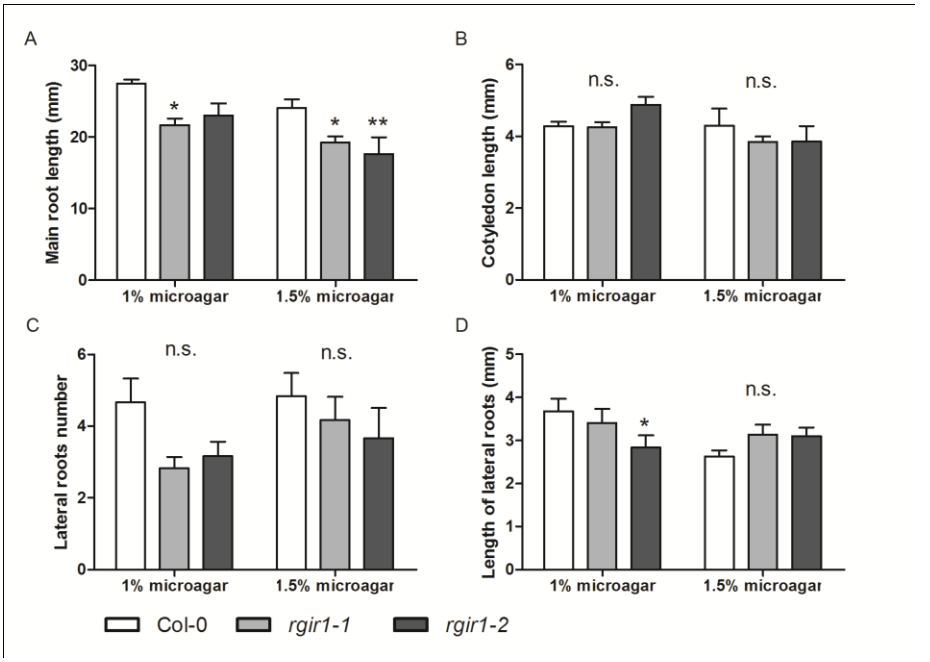

Figure 1. Effects of hard agar on root system architecture of *rgir1* mutants and wild type of *Arabidopsis thaliana*. Seeds of *rgir1* mutants and wild type Columbia were directly germinated on control medium (1/2 MS, 2.5 mM MES, and 1% micro-agar) and hard medium (1.5% micro-agar), and images of 7-d-old seedlings were scanned by a scanner (Epson scanner). Root length **(A)** and cotyledon length **(B)** were analysis with image software of WinRihzo connected to the scanner, and lateral root number **(C)** and average of lateral root length **(D)** were used to analysis effect of hard agar on root branching. Data are means ±SEM of six roots for each genotype. '*' represents significant difference at 0.05, and '**'' represents significant difference at 0.01 (two-way ANOVA).

Sucrose induces wavy phenotype of root grown on agar independently of root length and lateral roots changes

Root growth and wave amplitudes were analyzed for each mutant line to quantify the differences between wild type and *rgir1* mutants in response to growth on the agar medium with sucrose. Under control condition, roots of *rgir1-1* plants showed significantly shorter ($P<0.05$) main root length and lateral root length, whereas the number of lateral roots and the ratio of lateral root length to main root length was not affected. However, roots of *rgir1-2* were indistinguishable from wild type, as expected (**Figure 2 B**). In the presence of sucrose, roots of *rgir1-1* and *rgir1-2* also displayed wavy growing character like wild type (**Figure 2 A**). Waves of *rgir1-1* and *rgir1-2* roots have shorter wave lengths than wild type roots on sucrose medium, and they have more waves near the tip of the root, in the region where no lateral roots are observed. The precise function of RGIR1 in waving formation, however, is not known.

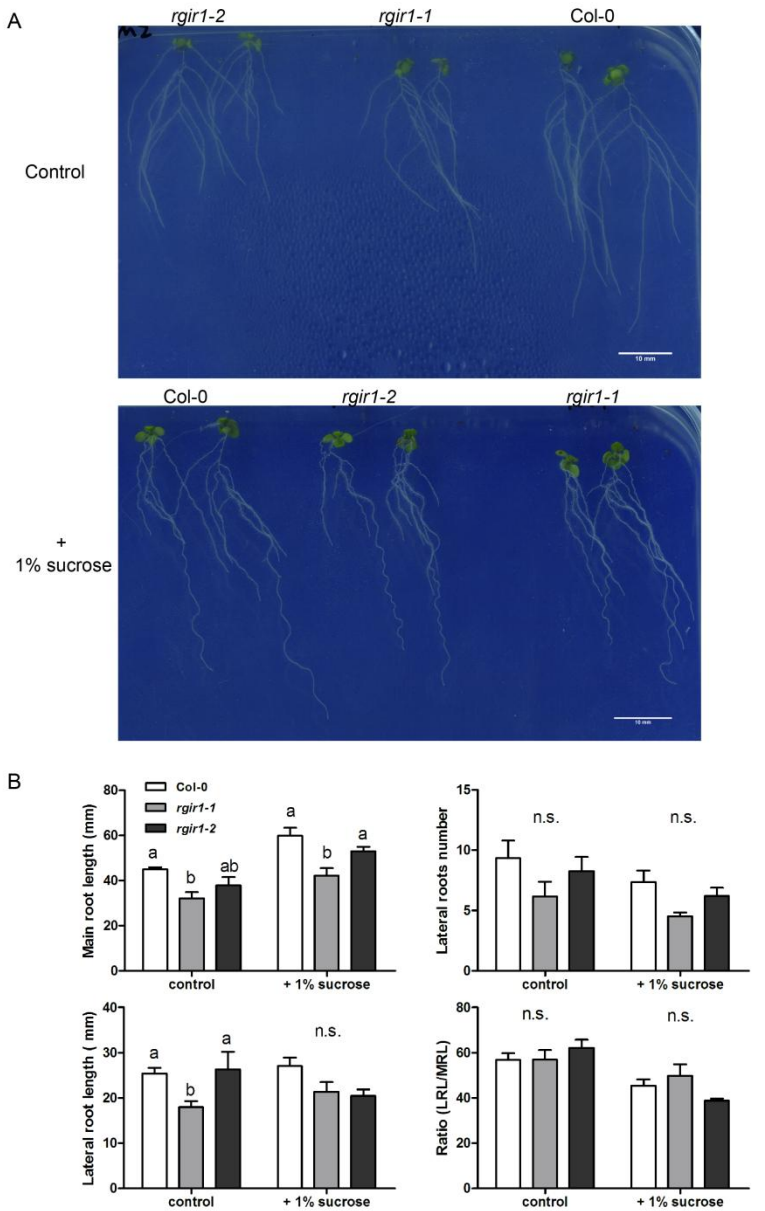

Figure 2. Response of root system architecture of wild type and *rgir1* mutants grown on hard medium supplied with 1% sucrose. A: 14-d-old root phenotype of *rgir1* mutants and wild type grown on control medium (1/2 MS, 2.5 mM MES, and 1.5% agar) with 1% sucrose and without sucrose. Scale bar = 10 mm. **B:** Length of primary root, total number of lateral roots, and the average length of lateral roots in wild type and *rgir1* mutants seedlings shown in (A). Data are Mean ±SEM of six roots for each phenotype. Different letters on top of the column represent significant differences between genotype under the same treatment condition ($P<0.05$, two-way ANOVA).

Repression of root elongation by NaCl is caused by the high ionic strength of the media

Arabidopsis is a salt-sensitive plant species (Munns and Tester 2008). Increasing the salt concentration in the media results in shorter main root length in both the wild type and *rgir1* mutant seedlings (**Figure 3 A**). Sucrose (1%) promotes main root elongation and inhibits lateral root formation when growing on the surface of a solid medium (**Figure 2**), but does not prevent the inhibition of root elongation by NaCl (neither in wild type, nor in the mutants). The effect of sucrose on root elongation and root branching is independent of the negative effect of salt on growth and is not differentially regulated in the *rgir1* mutants.

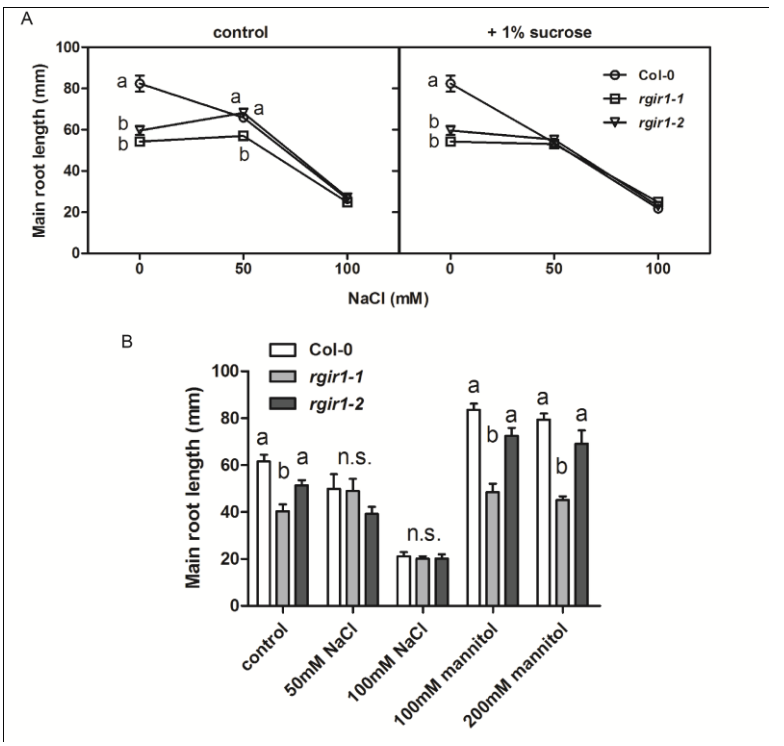

Figure 3. Responses of *rgir1* mutant plants and wild type plants to different concentrations of NaCl or mannitol. A: Growth (over a period of 12 days after germination) of wild type and *rgir1* mutants on the medium supplemented with different levels of NaCl (left, control) and medium supplied with 1% sucrose in addition to NaCl (right, + 1% sucrose). Data are mean ± SEM of 6 plants for each genotype. Different letters represent significant difference between genotype at the same level of NaCl ($P<0.05$, two-way ANOVA). **B:** 14-d-old root length of *rgir1* mtuants and wild type grown on hard (1.5 %) MS medium (1/2 MS, 2.5 mM MES, and 1% sucrose) with salt or mannitol. Values are mean ± SEM of 6 plants. Different letters on top of bar columns represent significant difference while n.s. indicates no difference was found between genotypes at the same treatment condition ($P<0.05$, two-way ANOVA).

To determine whether the repression of root elongation was caused either by the high osmotic value or the toxic ionic (Na^+ and Cl^-) effect or both, we supplemented the media with osmotically equivalent concentrations of mannitol. Mannitol appeared to have no effect on root elongation (**Figure 3 B**), indicating that the repression of root elongation on salt media is caused by the ionic effects. *rgir1-1* displayed significantly shorter ($P<0.05$) main roots on control media, but this difference disappeared both on mild and high salinity treatment media. Although both salinity and *RGIR1* mutation repress root elongation for *Arabidopsis*, salt does not induce a further reduction of root growth in *rgir1-1*, possibly indicating an interaction of salinity with gene function or suggesting that a further reduction of growth in the *rgir1-1* background by salt is not feasible.

Effects of Sulfur deficiency on root system architecture

In *Arabidopsis* seedlings grown on the surface of agar plates without SO_4^{2-}, lateral roots formed closer to tip of the root and developed at a higher density, compared to those grown on sulfate-sufficient medium (Lopez-Bucio et al. 2003). To assess the effects of sulfur deficiency on root architectural traits of wild type and *rgir1-1* and *rgir1-2* mutants, we first grew seedlings on control medium (+S) for 9 days and then transferred to new medium with (+S) or without (-S) sulfate for 16 days. After 7 d growth on the new medium, the primary root growth rate of wild type and the two *rgir1* alleles were not affected on the S-free medium (**Figure 4 A and B**). Root growth of *rgir1-2* was indistinguishable from wild type both on the +S medium and –S medium. However, the *rgir1-1* mutant displayed a 21% (**Figure 4 A**) and 41% (**Figure 4 C**) reduction in main root length after 7 d and 16 d growth on new +S medium. The reduction of main root length in *rgir1-1* was 22% and 49% for 7 d and 16 d growth on -S medium, respectively, suggesting that sulfur does not interact with RGIR1 in controlling elongation of root.

As shown in **Figure 4 E**, we carefully examined the formation of lateral roots in the tip part of primary root in both the segment that formed on sulfur-sufficient medium before transfer to new medium and the segment that was newly formed while on the new +S or –S medium. After 7 d growth on the new medium, lateral root formation was significantly increased by sulfur deprivation in both wild type and *rgir1* mutant roots (**Figure 4 D**). Although there were small differences between *rgir1-1*, *rgir1-2*, and wild type, these were not statistically significant. The density of lateral roots formed on the upper (proximal) segment was around 2-fold higher than that of distal segment that formed on the new medium in wild type and mutant seedlings (**Figure 4 F**). This pattern was identical in +S and –S roots. Since the number of lateral roots in all genotypes is nearly the same, but the root length is shorter in the *rgir1-1* mutant, the lateral root density in this mutant is significantly higher.

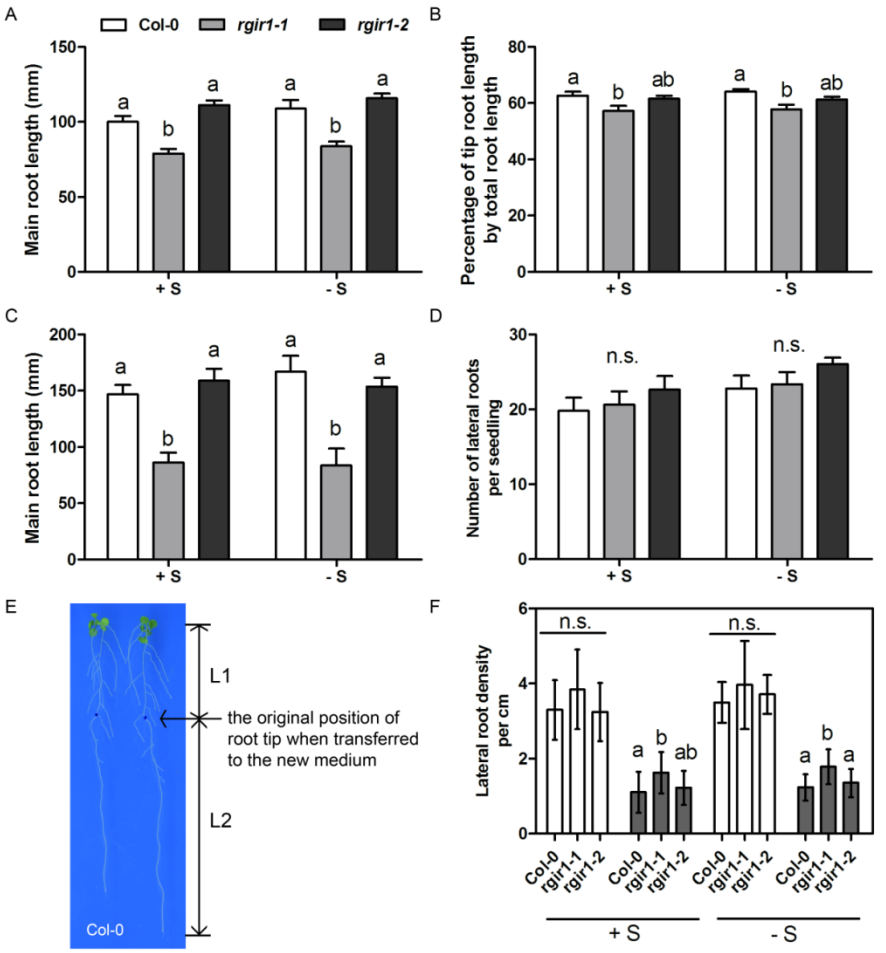

Figure 4. Effect of sulfur deficiency on root morphology of *rgir1* mutants and *Arabidopsis* ecotype Columbia. Seedlings were cultured for 9 d on 25% Hoagland (NO_3^-) medium solidified with 1% micro-agar and then transferred to medium without/with S for a further 16 days. **A:** Total length of main root of wild type and *rgir1* mutant seedlings grown on the new medium with or without S for 7 days. **B:** Percentage of length for the newly formed root segment on the new medium by total length of the primary root after 7 d growth on the new medium (+S or -S). **C:** Total length of main root of wild type and *rgir1* mutant seedlings grown on the new medium with or without S for 16 days. **D:** Total number of lateral roots in wild type and *rgir1* mutant seedlings grown on the new medium with or without S for 7 days. **E:** Schematic of root (wild type) grown on the Sulfur-sufficient medium (before transfer to fresh medium) and the new medium with sulfate or without sulfate for 7 days. The black dot on the primary root indicates the original position of root tip when transferred to the new medium. **F:** Lateral root density in different root segment (light bars for segment L1, grey bars for segment L2) in wild type and *rgir1* mutant seedlings on +S and –S medium. Values are mean ± SEM, n=6-18. Different letters on top of bar column in all graphs indicate significant difference between genotype at the same treatment condition ($P<0.05$, two-way ANOVA).

Response of roots morphology on low pH medium

Root growth and branching of both wild type and *rgir1* mutants were markedly reduced by lowering the pH of the growth medium from 5.7 (**Figure 5 A**) to 4.6 (**Figure 5 B**). Consistent with previous results, *rgir1-1* had a shorter main root compared to *rgir1-2* and wild type after 5 d incubation at pH 5.7, but this difference disappeared when grown at pH 4.6 (**Figure 5 C**). Seedlings of *rgir1-1* that were 16 days old showed more lateral roots than *rgir1-2* and wild type at pH 5.7. Low pH strongly reduced lateral root density and the difference among wild type and mutants plants was no longer visible (**Figure 5 D**). The ratio for the newly formed root on the new medium of *rgir1* mutants were similar with wild type Col-0 after 5 days grown on the control medium, but it was significant decreased in the *rgir1-1* seedling compared with Col-0 and *rgir1-2* at the same medium (**Figure 5 E**). The decrease of ratio in *rgir1-1* was even enlarged in the prolonged culture period on the fresh low pH medium (**Figure 5 F**), indicating that low pH exacerbated the function of RGIR1 in controlling root growth of young seedlings.

Discussion

Plant growth is a complex and highly dynamic process, and the root system of plants shows a high plasticity in growth and development in response to environmental change (Sánchez-Calderón et al. 2013; Ryu and Cho 2015; Petricka et al. 2012; Scheres et al. 2002; Walter et al. 2009). Our data reveal that the surface-dependent growth behaviors of root were easily influenced by slight changes in the (nutrient) properties of the medium.

Without sucrose, the wavy phenotype was not observed in wild type Columbia seedlings and no difference was found between roots grown on standard medium (1% agar) and on hard agar with 1.5% micro-agar (**Figure 1**). While seedlings showed approximately straight root tip growth and had increased branching angles when grown on the control medium without sucrose (**Figure 2**), vertically grown wild type Col-0 seedlings displayed a waving phenotype combined with right skewing in the presence of sucrose. In addition, sucrose (1%) induces a substantial increase in main root growth and a decrease in the formation of lateral roots.

A stress-induced morphogenetic response in roots consists of inhibition of root elongation and an increase or decrease in lateral roots number (Potters et al. 2007). *Arabidopsis* is a relatively salt-sensitive species compared to other species, such as rice, wheat, and barley (Munns and Tester 2008). Treating *Arabidopsis* wild type Columbia with salt inhibits root elongation at relatively low concentrations of NaCl (**Figure 3 A**) (see for instance also **Zolla et al. 2010**). Root system architecture response to stress requires coordination of many genes and an intricate signal transduction network of receptors, second messengers and transcription factors (Scheres et al. 2002; Shiu and Bleecker 2001; Stahl and Simon 2012). Stress responses in RSA are tightly coordinated with specific developmental processes and some stress responses appear to show cell-type-specificity (Iyer-Pascuzzi et al. 2011; Dinneny et al. 2008). The *rgir1-1* mutants display a smaller root system with a

shorter main root length and less lateral roots when cultured on standard MS medium without sucrose (**Figure 1**). In response to sucrose and salt, roots of *rgir1-2* were indistinguishable from wild type on the same plate, but root elongation of *rgir1-1* does not seem to be inhibited further by salt stress (**Figure 3**).

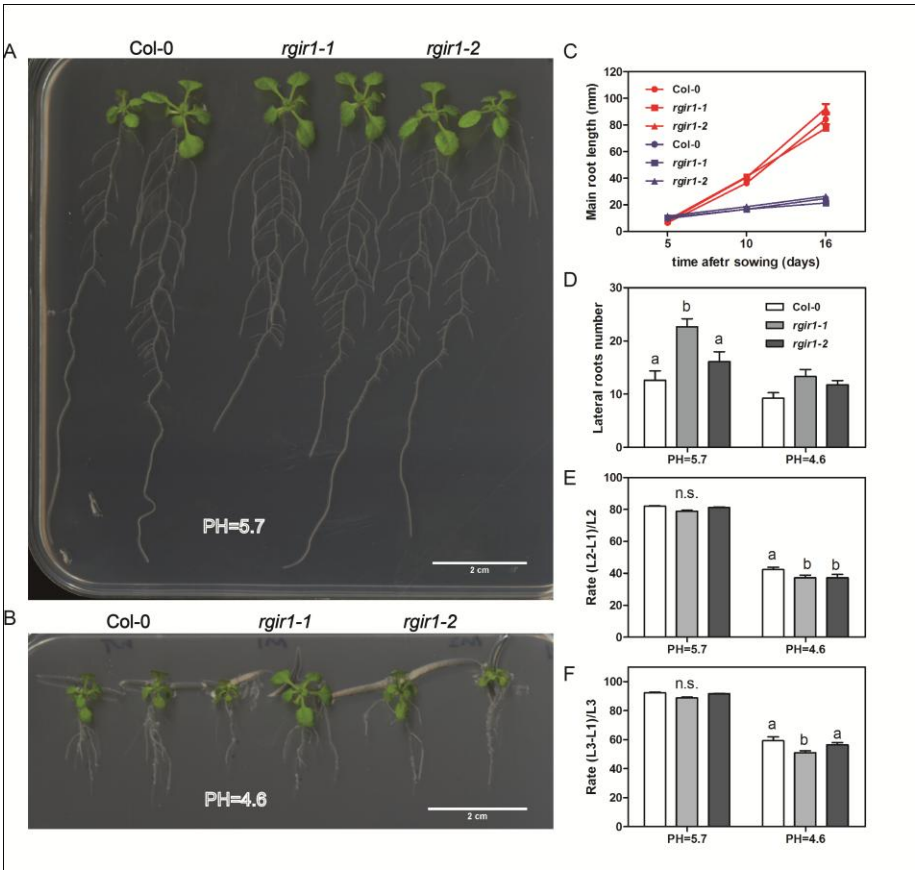

Figure 5. Responses of root morphology on medium with low pH. A-B: Root phenotype of 16-d-old *rgir1* mutants and wild type seedlings grown on control medium (pH=5.7, A) and low pH medium (pH=4.6, B). **C:** Growth of main root in wild type *rgir1* mutants seedlings grown on medium with pH 5.7 (red line) and 4.6 (blue lines). Value are mean±SEM, n=12. **D:** Lateral roots number of 16-d-old seedlings in *rgir1* mutants and wild type grown on control medium and low pH medium. **E-F:** Ratio of the length of newly formed root on the new medium for 5 days (E) and 11 days (F) to the total root length of plants under control medium and low pH medium.Value are mean±SEM, n=12. Different letters on top of bar column indicate significant difference between genotype at the same treatment condition ($P<0.05$, two-way ANOVA).

At low pH expression level of *rgir1* was increased in the transition zone of wild type root tip stress (Iyer-Pascuzzi et al. 2011) and the main root elongation was suppressed significantly, both in *rgir1-1* mutant and wild type plants. The *rgir1-1* mutant had more lateral roots than wild type at both pH 5.7 and 4.6, regardless of the difference in growth rate of the main root. Therefore, despite the function as a

growth regulator in the root elongation zone RGIR1 might also play a role in lateral root induction.

Nutrient availability exerts a profound impact on root system architecture and plant roots exhibit strong morphological responses to different deficiencies (Gruber et al. 2013; Hodge 2004). For instance, primary root length is significantly decreased when grown under low P and K in the medium, but is slightly increased under intermediate supplies of N and Fe (Zhang and Forde 2000; Williamson et al. 2001). Nutrient deficiencies also induce changes in lateral root initiation (Gruber et al. 2013; López-Bucio et al. 2002). *Arabidopsis* wild type seedlings produce a highly branched root system with abundant lateral roots and a shorter primary root at low P, K and S concentration medium (López-Bucio et al. 2002; Williamson et al. 2001; Kutz et al. 2002), whereas the lateral root density is not affected under N and Ca deficiency (Gruber et al. 2013). We observed that primary root length of wild type was not affected by -S, but the lateral root formation was markedly increased (**Figure 4 C and F**). Apart from the genetic control of the internal gene network of plants, the total number of lateral roots depends on environmental factors (Malamy and Ryan 2001). Expression of RGIR1 was strongly enriched in the endodermis (Iyer-Pascuzzi et al. 2011), indicating a possible role for RGIR1 in controlling lateral root initiation in the pericycle, the cell layer immediately inside the endodermis. Indeed, the lateral root density of *rgir1-1* plants was higher compared to wild type and *rgir1-2* both under control medium and the –S medium. It remains to be confirmed that this increased lateral root density is the result of stimulated root initiation or an effect of main root growth inhibition.

Chapter5

Salt stress and root-agar interactions affect the root skewing Behavior in *Arabidopsis*

Nana Yu, J. Theo. M. Elzenga

Laboratory of Plant Ecophysiology, Groningen Institute for Evolutionary Life Sciences, University of Groningen, The Netherlands.

Abstract: *Arabidopsis thaliana* roots display waving and skewing growth patterns when grown on inclined medium, and the directional growth of roots is modulated by endogenous factors (e.g. circumnutation) and various environmental signals (e.g. gravity, water and nutrients in the medium). This work showed that the presence of sucrose and salt in the medium extensively modulates directional root growth in *Arabidopsis* seedlings. When grown on vertical agar medium, Col-0 displayed rightward slanting roots and this slanted phenotype was enhanced in the presence of 1% sucrose. However, roots of Col-0 and *rgir1* mutant seedlings developed anticlockwise root coils on horizontal medium and a hooked root tip when culture plates were inclined at an angle of 45°. High salt stress altered root directional growth combined with severe suppression of main root length and lateral roots formation in wild type and mutants, probably caused by uptake of sodium, but not osmotic stress. In addition, high salinity (100 mM NaCl) induced strong right-hand helical growth in wild type and mutants seedlings. Both loss of function mutant *rgir1-1* and knock-down mutant *rgir1-2*, displayed indistinguishable phenotypes in the root direction growth when compared with wild type. Thus, sucrose and salt are key regulators in the alteration of root surface growth of *Arabidopsis* roots, and RGIR1 seems not involved in the root directional growth on the surface of the solid medium except its role in controlling root elongation in the transition zone of the root tip.

Introduction

A common way to track root growth behavior and root system architecture of plants is to germinate seedlings in a vertically placed petri dish on agar supplied with nutrients. Primary roots of plant display a characteristic surface-growth pattern that comprises of waves, coils and slanting towards one side from the gravity vector, especially when the plate is inclined at 30 °to 60 °from the vertical plane (Migliaccio and Piconese 2001; Mirza 1987; Simmons et al. 1995a; Simmons et al. 1995b; Okada and Shimura 1990). Based on three-dimensional images and genetic analyses of skewing/waving mutants, all these movements are assumed to be the result of the interaction between positive gravitropism and negative thigmotropism, together with the process of circumnutation, a more commonly found process in the above ground parts of plants (Migliaccio et al. 2013; Migliaccio and Piconese 2001).

Plant organs display helical growth movements known as circumnutation and produce tracks that are commonly elliptical or circular (Migliaccio et al. 2013; Kitazawa et al. 2005; Kiss 2006). Roots of *Arabidopsis* seedlings grow in a circle when the force of the gravity is excluded (i.e. in a Space Station) (Johnsson et al. 2009; Scherer and Pietrzyk 2014), but exhibit an oscillatory pattern called waving under earth's gravity when grown on inclined agar plates (Migliaccio and Piconese 2001; Migliaccio et al. 2013). This waving pattern was first published as being the result of thigmotropism by Okada and Shimura (1990, 1992). They hypothesized that roots grown on an inclined mediumsense both a touch experience and gravity and the formation of waves would be the result of negative thigmotropism of the root tip with agar surface and the positive gravitropism of plants. However, Simmons et al. (1995) argued that this waving growth patterns results from the interaction between the intrinsic circumnutation and gravitropism. A recent idea considers the circumnutation of little importance in the formation of root waving, assuming that the interaction between gravitropism and root tip impedence is sufficient for generating the waving/coiling phenotype (Thompson and Holbrook 2004). Other factors that are considered in this still open debate on the establishment of waving or coiling phenotype are: possible involvement of hormone (s) (Vanneste and Friml 2009), light (Oyama et al. 1997) and anisotropic cellular growth (De Smet et al. 2007).

In addition to waving and/or coiling, roots tend to deviate their growth away from the gravity vector, typically rightward for most *Arabidopsis* ecotypes when observed from the back of plates, through the agar (Migliaccio and Piconese 2001; Oliva and Dunand 2007). When grown on titled agar surfaces, the degree of slanting angle differs in ecotypes of *Arabidopsis* plants, with little or no slanting for Columbia and more slanting to the right for *Landsberg* and *Wassileskija* (Migliaccio and Piconese 2001; Migliaccio et al. 2013). Recently, mutants have been discovered that differ in the root waving and skewing patterns on the inclined hard-agar plates (Migliaccio et al. 2013). The *wav1-1* mutant produces mostly straight root growth with few waves on a titled plate (Okada and Shimura 1990), whereas, the *wav2-1* and *wav3-1* mutant display enhanced wavy root growth on inclined agar surface with shorter-pitch

waves in their roots (Mochizuki et al. 2005; Sakai et al. 2012). Other mutants were identified that had enhanced right skewing (i.e. *sku1, 2* or *lefty1, 2*) or a pronounced leftward skew (i.e. *spr1, 2* or *wvd2*) (Rutherford and Masson 1996; Abe et al. 2004; Thitamadee et al. 2002; Furutani et al. 2000; Yuen et al. 2003).

Many skewing mutants also display epidermis cell file rotations (CFRs) and abnormal cortical microtubule array formation. In fact, re-arrangement of the cytoskeleton could be an important process for epidermis CFRs and twisted growth of plants (Ishida et al. 2007). At the molecular level, genes affecting skewing are mainly involved in the arrangement of cytoskeleton structure, such as *left1* and *left2* (Thitamadee et al. 2002), SPR1 and SPR2 (Furutani et al. 2000), which encode proteins that correlated with stability of the cytoskeleton in the epidermal cells. The *Arabidopsis sku* mutant seedlings show an exaggerated right skewing phenotype on agar (Rutherford and Masson 1996; Sedbrook et al. 2002; Sedbrook et al. 2004). Cloning of genes of *sku6* and *spr1* revealed that they are the same gene, and *sku6/spr1* encodes a plus end-localized microtubule interacting protein that is involved in directional expansion of cell walls (Sedbrook et al. 2004). In addition, microtubule-depolymerizing molecules, such as propyzamide, oryzalin and taxol, or microtubule-stabilizing compounds can provoke or suppress skewing (Sedbrook et al. 2004; Furutani et al. 2000), clearly supporting the effect of re-arrangement of the cytoskeleton on root skewing and waving.

The mutant WAVE DAMPEND 2 (*wvd2)* roots skewed leftwards without waving when grown on inclined agar with respect to its wild type ecotype (Nossen-0) (Yuen et al. 2003), indicating that different processes regulate the waving and skewing behavior of roots. Compared with skewing, the genes affecting waving seem to have different functions. Most mutants discovered with abnormal waving phenotypes were identified to be related to the gravitropic response. The first discovered waving mutants *(wav1-6*) were almost all defective in the root gravitropic response (Okada and Shimura 1990). Among them, some of the mutations were further identified as allelic with genes mediating influx (*wav5/aux1*) and efflux (*wav6/pin2*) of the plant hormone auxin (Müller et al. 1998; Rashotte et al. 2000). Auxin appears to be involved in most of the tropic reactions studied, including gravitropism (Reviewed by Vanneste and Friml 2009; Kleine-Vehn and Friml 2008; Rashotte et al. 2000; Muday et al. 1993). De Smet et al. (2007) reported that application of the auxin transport inhibitor naphthylphthalamic acid (NPA) blocks root waving and the gravitropic response. Furthermore, the hybrid *wag1/wag2* double mutant develops pronounced and compressed waves even on vertical gar plates and its root are more sensitive to NPA in the root curing processes (Santner and Watson 2006). Although the precise functions of these genes are still lacking, they seem to mainly affect the perception of gravity and have roles in transducing the gravity signal to produce waves.

Recently, it was found that other hormones (i.e. ethylene, cytokinin) and the contact between the root and medium also play important roles in the process of waving and skewing (Buer et al. 2003; Thompson and Holbrook 2004). While our

study of the loss function of gene in the *rgir1* mutants was in progress, we found that roots grown on the vertical medium always skewed to one side of the plate and changed growth direction in the absence of osmotically active compounds in the medium. The goal of this study was to assess whether RGIR1 is involved in controlling the directional growth of the roots and how salt and growth medium affect the root waving and slanting behavior on agar surfaces. Our results show that roots display waving patterns and coils on tilted growth medium at different angles, and that sucrose enhances the extent of skewing of roots on vertically placed agar plates with control medium without other osmotic compounds. Furthermore, we have also found that treatment of seedlings with NaCl, besides inhibiting root elongation, induces leftward skewing (observed from the bottom of plate through the agar) at higher concentration and that this NaCl-induced change of growth direction is not due to the osmotic change alone. In the phenotypic analysis of *rgir1* mutants, no significant difference was found compared with wild type plants that were treated the same, indicating that RGIR1 mainly affects root elongation, but is not involved in controlling the direction of growth. Taken together, these results demonstrated that sucrose and salt are key regulators in the alteration of root surface growth patterning of *Arabidopsis* roots.

Material and methods

Plant materials and growth conditions

Arabidopsis thaliana wild type (Col-0) and T-DNA insertion mutants, *rgir1-1* and *rgir1-2,* were utilized to study the waving and skewing phenotype in this work. F1 seeds of wild type and homozygous mutants harvested at the same time were first surface sterilized as described in chapter2, and then were sown on the surface of 5 mm thick medium containing 1/2 MS, 2.5 mM MES, 1% sucrose, and 1% micro-agar, unless otherwise noted. Each plate was sealed with parafilm tape and seeds were stratified on the plate for 3-4 days at 4 ℃. Subsequently, plates with seeds were transferred to a growth chamber at 21 ℃. Light of the chamber was provided by red/blue fluorescent lights with an average fluence rate of 120 μmol m^{-2} s^{-1} following a photoperiod of 16 hours light/ 8 hours dark.

Effect of light, sucrose, and inclined medium on root skewing phenotype

Seeds were germinated on the plates that were directly placed vertical (θ=90°), horizontal (θ=0°) or titled at a 45° angle. In order to study the effect of light on the skewing root phenotype, one group of plates were placed vertically and covered by a black box. For experiments testing the effect of sucrose on root directional growth, seeds were germinated directly on 1/2 MS medium with 2.5 mM MES, 1% or 1.5% micro-agar, and with or without 1% sucrose, and then placed vertically in the same chamber at 21 ℃. Images of roots were taken though the medium from the back of the plate by a scanner (Epson scanner), and Day0 of the growth is defined as the time when plates were transferred to the 21 ℃ growth chamber.

NaCl-induced root phenotype

In all NaCl exposure experiments, imbibed seeds were grown and germinated on square petri dishes (12×12 mm) containing 1/2 MS medium buffered with 2.5 mM MES, supplemented with 1% sucrose and 1% micro-agar, unless otherwise noted. All plates were sealed with parafilm tape and placed vertically in a growth chamber at 21 ℃ during day and night with a photoperiod of 16 hours light/8 hours dark and 75% relative humidity. For experiments testing the effect of salt on the skewing phenotype of root, the indicated concentrations of 0, 50, and 100 mM NaCl were used to supplement the standard medium, which already contains 0 mM Na^+ and 6.2 mM Cl^-. To examine whether salt-induced directional growth of the root was caused by a general osmotic stress or by the presence of Na^+ and Cl^- ions, the equivalent concentrations (100 and 200 mM, respectively) of mannitol medium in 1.5% micro-agar, was used as a control.

Measurements of roots

ImageJ (https://imagej.nih.gov/ij/) was used to determine parameters according to the method described before (**Figure 1**) (Grabov et al. 2005; Vaughn and Masson 2011). The length of the primary root (L) is measured by tracking the root with the "segmented line" tool; vertical growth index (VGI) is the ratio of displacement of root tip Ly divided by the root length L, and similarly displacement of root tip Lx divided by L gives the horizontal growth index (HGI). The root deviation was measured by taking the angle α from the vertical axis (0°), assigning a positive sign when moving counterclockwise and a negative sign when moving clockwise. Taken together, VGI, HGI, and the deviation angle α enable quantification of skewing growth pattern of root under different treatment.

Results

Sucrose enhances the rightward skewing phenotype of *Arabidopsis*

As shown in **Figure 2 A**, roots of *Arabidopsis* wild type skewed to the right on vertically placed plates with 1% micro-agar medium when observed from the bottom of agar plated through the agar. The slanting wild type roots was suppressed when we increased the micro-agar concentration from 1 percent to 1.5 percent in the absence of sucrose, whereas, the rightward slanting was enhanced in the presence of 1% sucrose both on standard medium (1% gar) and hard agar (1.5%). We next quantified the skewing phenotype by measuring the slanting degree of the root tip from the vertical axis measured from the point of connection between root and stem (**Figure 1 B**). We found that, the rightward slanting angle (α) in wild type roots was reduced by 61% when grown on hard agar (1.5%) compared to 1% agar medium (**Figure 2 B**). Including 1% sucrose in the medium increased the slanting angle by 100% both for standard medium and hard agar. In the presence of 1% sucrose, the rightward slanting angle (α) was increased almost two-fold, compared with control medium with 1% agar. However, the sucrose induced enhancement of rightward slanting was strongly reduced on hard agar, possibly caused by altered friction between the root tip and the hard agar.

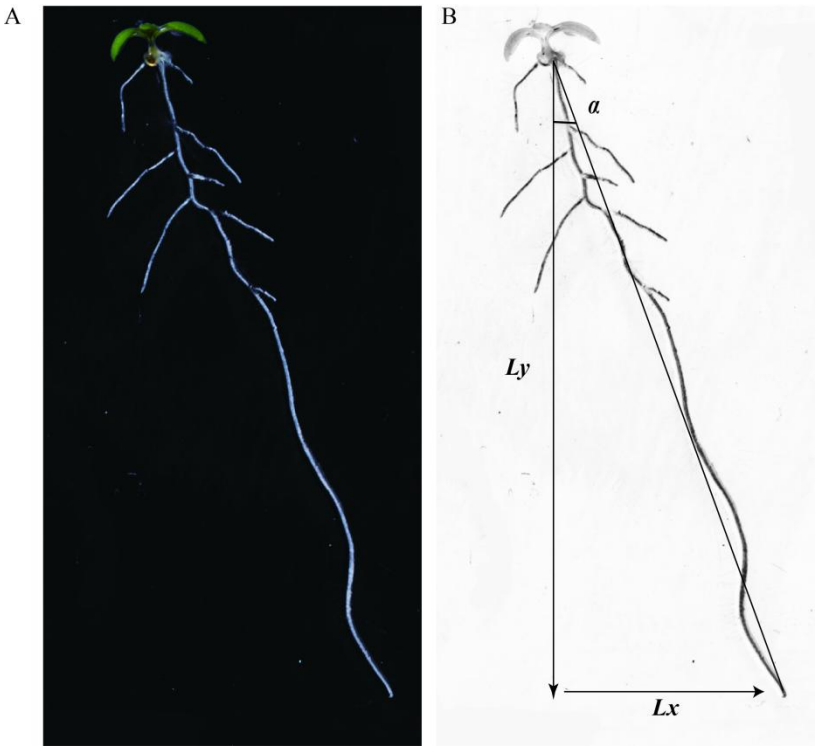

Figure 1. Quantification of root skewing phenotypes of *Arabidopsis* seedlings grown on agar medium. A: 7-d-old seedling of *Arabidopsis* ecotype Col-0 grown on vertical 1/2 MS medium. **B:** Measurement of parameters describing skewing: α, skewing angle; Ly, displacement of the root tip along the y-axis; Lx, displacement of the root tip along the x-axis. The total length of the primary root (L) is calculated by tracking the root with "segmented line" of ImageJ, and the vertical growth index (VGI) is given by the ratio of length of Ly in total root length. Similarly, length of Lx in a ratio of L gives horizontal growth index (HGI). The skewing angle is calculated directly by the "angle tool" function of ImageJ.

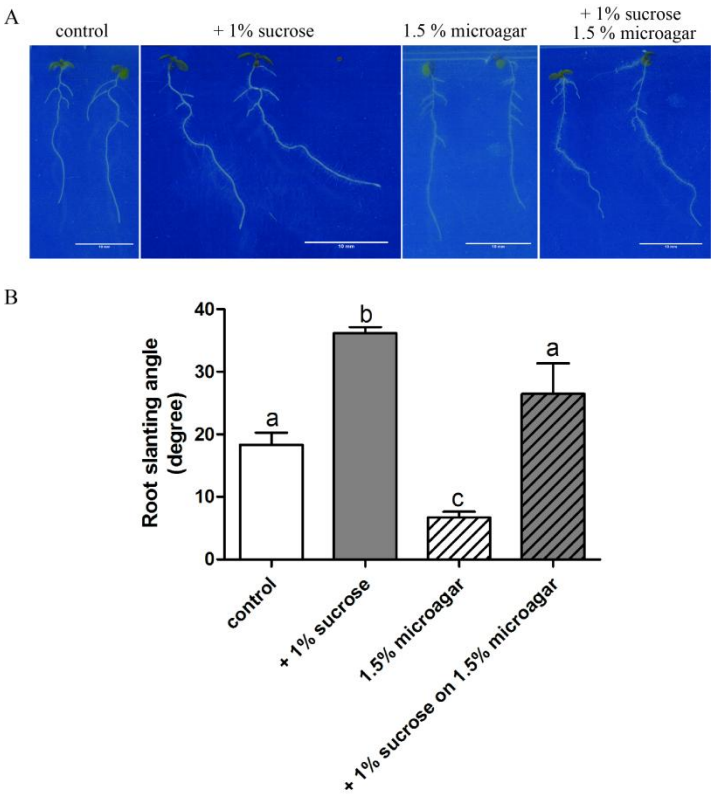

Figure 2. Effect of exogenous sucrose and hard agar concentration on the rightward skewing phenotype of *Arabidopsis* ecotype Columbia. A: 7-d-old seedling of wild type (Col-0) grown on control medium with 1/2 MS, 2.5 mM MES and 1% micro-agar and treatment mediums supplied with 1% sucrose or with higher concentration (1.5%) of micro-agar. Images were taken from the back of the plate through the agar. Scale bar = 10 mm. **B:** The root slanting angle of root tip in 7-d-old Col-0 seedling deviated from vertical. Data are mean + s.e.m. of three biological replicates with 6 to 21 seedlings. Different letters on top of the bar columns indicate significant difference between treatments ($P<0.01$, Tukey's Multiple Comparison test).

Root coils and skewing phenotypes on tilted medium

To demonstrate the effect of inclining the angle of the growth medium on root skewing phenotype, agar plates were places at three different inclination angles from the gravity vector (0°, 45° and 180°). At 0° (vertical), roots of wild type seedlings skewed to the right and formed waves in the primary root (**Figure 3 A**). *rgir1-1* seedlings displayed enhanced, but not statistically significant, rightward slanting on the vertical media compared with wild type, whereas, the rightward slanting angle (α) was significantly decreased in *rgir1-2* seedlings compared with *Arabidopsis* ecotype Columbia (**Figure 3 B**). On plates with a 45° inclination angle the roots stopped waving and showed a slightly coiled phenotype, while on a horizontal surface (180°) roots continued to developed anticlockwise coils as seen from the bottom of the plate without waving (**Figure 3 C**). Waving and coil pattern of *rgir1* mutant seedlings

grown on tilted medium were similar as that of wild type (data not shown).

In an attempt to study the effect of light on the directional growth of root, seeds of wild type and *rgir1* mutants were germinated directly on vertical agar plates covered by a black box. Waving movement of dark-grown seedlings was similar to those of light-grown seedlings of wild type on the vertical plate in the same growth chamber (**Figure 3 D**). However, the rightward slanting was reduced in the darkness and seedlings developed shorter roots and longer hypocotyls compared with those grown under 16 hours light/8 hours dark photoperiod, both in wild type and mutant seedlings (data not shown).

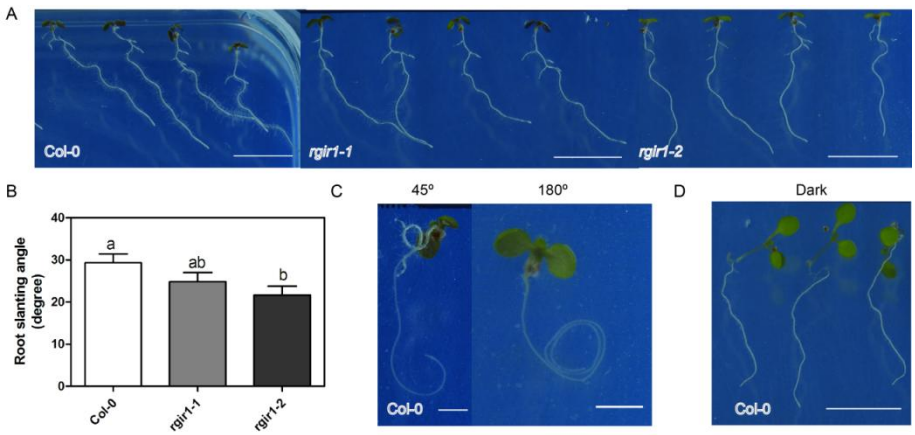

Figure 3. Root growth phenotypes of wild-type (Col-0) and *rgir1* mutants seedlings grown on titled mediums. Wild type (Col-0) and *rgir1* mutant seedlings grown 7 d on 1% agar-solidified 1/2 MS medium placed vertically **(A)** or tilted at 45 °and placed horizental **(C)** in the growth chamber with light period of 16 hours light / 8 hours dark. **B:** The slanting angle of wild type and *rgir1* mutants seedling deviated from vertical y-axis that shown in (A). Data are mean + s.e.m, n=29. Different letters on top of the bar columns indicate significant difference between genotypes (P<0.05, Tukey's Multiple Comparison test). **D:** Wild-type (Col-0) seedlings grown 9 d on 1% agar-solidified 1/2 MS medium in the same chamber as (A) and (C), but in the dark. Note: all images were taken from the back of the plate, through the agar. Scale bar = 1 cm in (A) and (D), and 2 mm in (C).

NaCl induced root growth direction changes independently of root length change

When grown vertically on a hardagar (1.5% micro-agar) plates without salt, roots of wild type and *rgir1* mutants skewed to the right of the plate (**Figure 4 A**). In contrast to the rightward skewing on the control medium, roots grew almost straight and parallel to the vector of gravity, or even skewed to the left, when the medium was supplied with 50 mM NaCl in wild type and *rgir1* mutants (**Figure 4 B**). As shown in **Figure 4 C**, high salinity (100 mM NaCl) induced a strong right-handed helical arrangement of epidermal cell files (**Figure 4 D**), resulting in a more leftward skewing in wild type and mutants seedlings (**Figure 4 C**) compared with those on control medium (**Figure 4 A**) and 50 mM salt medium (**Figure 4 B**).

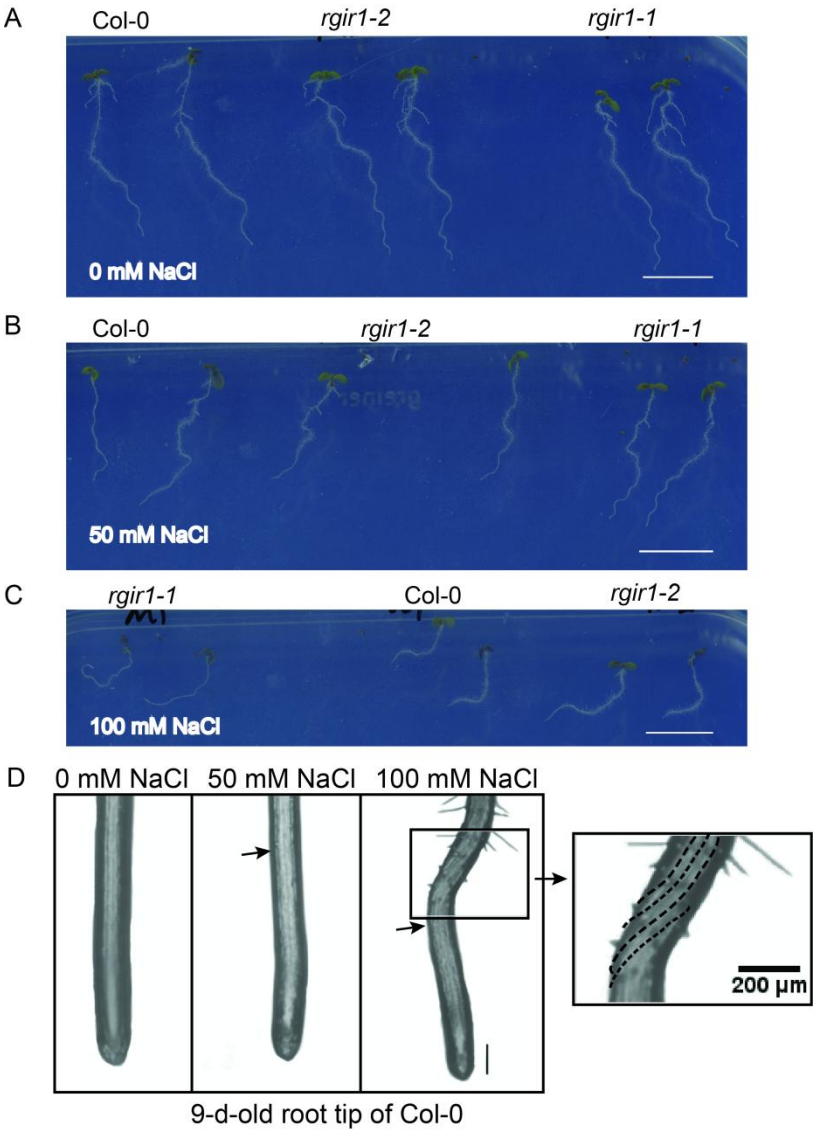

Figure 4. Root growth phenotypes of wild type (Col-0) and *rgir1* mutant seedlings grown on medium supplied with salt. Seedlings of wild type and *rgir1* mutants were germinated directly and grown 9 d on 1/2 MS hard medium (1.5% micro-agar) without salt **(A)** or with 50 mM **(B)**, and 100 mM NaCl **(C)**. Images were taken from back of the plate through the agar. Scale bar = 1 cm in A to C. **D:** Root tip phenotype of 9-d-old seedlings in Col-0 grown under different concentrations of NaCl. Arrows marked the first root hair bugle under 50 mM and 100 mM treatment and the twisting of the epidermal cell files under 100 mM salt treatment. Scale bar = 200 µm.

As shown by the root slanting angle and the primary root length results (**Figure 5 A and B**), the length of Col-0, *rgir1-1*, and *rgir1-2* was clearly suppressed in the presence of high salinity (100 mM) and the root slanting angle was also strongly exaggerated, but skewed to the opposite direction, when compared with growth

conditions without NaCl. When seedlings were grown on medium containing 50 mM NaCl, initially skewed root began to grow parallel to the vector of gravity or leftwards in wild type and mutant seedlings. While *rgir1-1* has the shortest primary root length in control medium and in 50 mM NaCl medium (**Figure 5 B**), the slanting angle of *rgir1-1* is similar to those in wild type and *rgir1-2* (**Figure 5 B**), indicating that RGIR1 only affects the elongation of the cells in root tip, but is not involved in helical growth of the root.

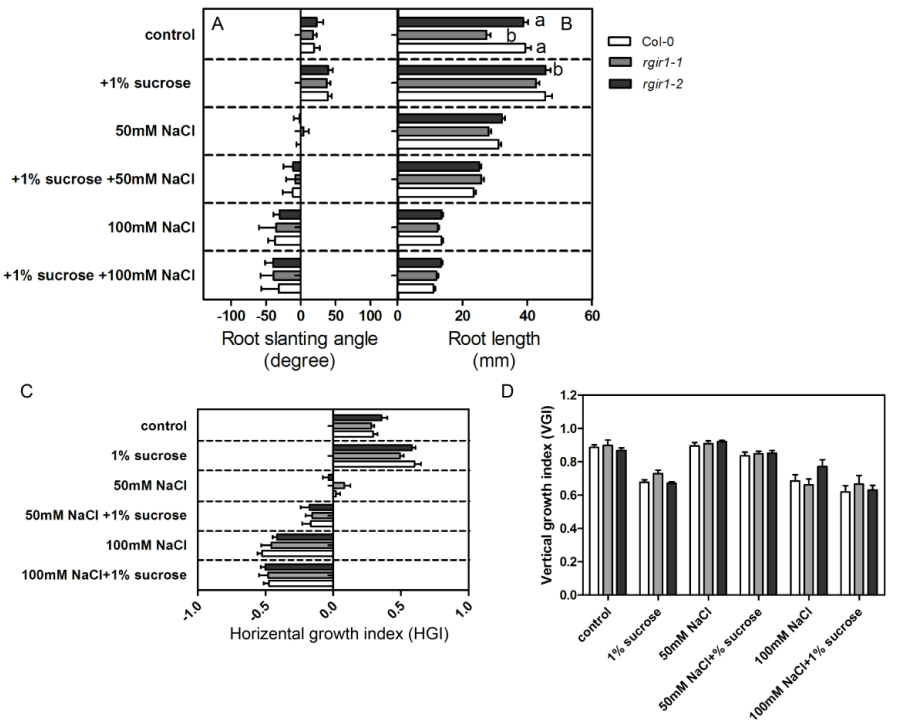

Figure 5. Quantification of root skewing phenotypes of wild type and *rgir1* mutant seedlings under salt treatment. Seedlings of wild type (Col-0) and *rgir1* mutants were grown for 9 days on salt medium without or with 1% sucrose. **A-B:** Effect of salt and sucrose on the root slanting angle (A) and main root length (B). Letters at the right side of bars in B indicate significant difference between genotype at the level of $p<0.05$ (n=13-16, Tukeys' Multiple comparison Test). **C-D:** Dynamics of root vertical growth index (VGI, C) and horizental growth index (HGI, D) in wild type (Col-0) and *rgir1* mutant seedlings under different treatments. No difference was observed between wild type and mutant seedlings for VGI and HGI at the same treatment condition.

In the presence of 1% sucrose in the medium, the seedlings show a pronounced rightward slanting with a concomitant increase in root length, both for wild type and mutants seedlings. When grown on salt medium, adding sucrose affects root elongation both at the lower (50 mM) and higher (100 mM) levels of NaCl in wild type and mutants seedlings. However, the salt-induced leftward skewing is enhanced

in the 50 mM medium in the presence of 1% sucrose, whereas it was not affected on the 100 mM medium.

The vertical growth index (VGI) and horizontal growth index (HGI) analysis is a versatile method for quantifying the deviation of root tip from straight downward vertical growth (Grabov et al. 2004). We next measured VGI and HGI to study the dynamics of root development in different medias. Although the root length and root slanting angles of Col-0 and *rgir1* mutant seedlings were different under different treatments (with or without sucrose; different concentrations of NaCl) we did not detect a significant difference in HGI and VGI of those seedlings when treated identically (**Figure 5 C and D**), which support the idea that VGI and HGI are independent parameters of root development processes (Grabov et al. 2004). When we only focus on the treatment effects, VGI in wild type and mutant seedlings was lower in 1% sucrose, 100 mM NaCl or 100 mM NaCl with 1% sucrose, compared with those grown on control medium (**Figure 5 D**). However, an increased was observed in HGI for those seedlings with lower VGI, indicating the roots tend to deviate to one side (left or right) from the vertical by positive HGI.

Compared with seedling treated with 100 mM NaCl, only 36% seedling skewed to the left when treated with 50 mM NaCl in wild type seedling, while that is 33% and 50% in *rgir1-1* and *rgir1-2* seedlings, respectively. In the presence of 1% sucrose in the 50 mM salt medium, the leftward skewing in wild type seedlings was increased to 85%, while it was 79% for *rgir1-1* and *rgir1-2* mutant seedlings. However, the VGI and HGI were not statistically different between wild type and mutants under treatment with 50 mM NaCl. As shown in **Figure 5 C and D**, the VGI in wild type and *rgir1* mutant seedlings on the 50 mM salt medium was not affected by adding sucrose, but HGI was significantly increased by including 1% sucrose in the medium. Thus, the sucrose-induced enhancement of root skewing is mainly caused by an increased growth rate and and not by an change in the skewing angle of root growth.

Salt-induced root growth direction changes are not merely due to osmotic stress

To determine which aspect of salt stress, osmolarity or ionic toxicity, affects the skewing phenotype, we monitored root growth of seedlings on medium containing osmotically equivalent concentrations of mannitol and of NaCl. When seedlings were grown in the presence of NaCl at different concentrations, the initial rightward slanting angle first decreased to 0 °and then changed further into a leftward slant in a dose dependent manner, with a concomitant decreased of root length, both in wild type and mutants seedlings (**Figure 6 A and C**). In contrast, neither the main root length nor the skewing angle was affected by osmotically equivalent concentrations of mannitol up to 200 mM (**Figure 6 B and D**). The HGI and VGI were not affected by mannitor in wild type or *rgir1* mutant seedlings (**Figure 6 F and H**), whereas, HGI of all genotypes were affected by NaCl (**Figure 6 E**). As expected, VGI of roots in all genotypes were slightly affected by 50 mM NaCl in the medium, but decreased strongly in 100 mM NaCl. The clear difference in effect of NaCl and mannitol indicates that the suppression of root length and salt-induced changes in the growth direction of the root is due to ionic toxicity of Na^+ or Cl^-, but not to osmotic stress in

the medium. Although *rgir1-1* seedlings showed a significant shorter root length in 200 mM mannitol and a lower VGI in 100 mM NaCl, it is too earlier to assume a role for RGIR1 in growth direction of roots.

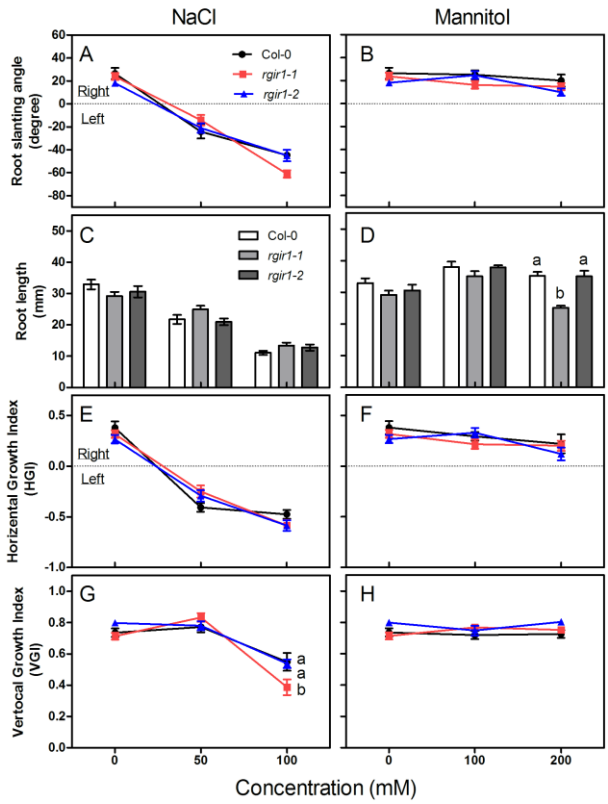

Figure 6. Effect of NaCl and mannitol treatment on root phenotypes in wild type (Col-0) and *rgir1* mutants. Seedlings of the indicated genotypes were grown for 9 d on a vertically placed hard agar medium (1/2 MS; 2.5 mM MES, 1% sucrose, and 1.5% agar) plate supplied with 0, 50, and 100 mM NaCl, or supplied with the equivalent concentrations mannitol. Data are mean ±s em with 6 to 7 seedlings per genotype. Different letters on top of bars in D or on the right of the scatters dot in G indicate significant difference between genotypes ($P<0.05$, Two-way ANOVA).

Discussion

Gravitropism and the root-agar interactions modulate root phenotype

The high plasticity of the root system allows plants to adapt to various external stimuli in order to maintain growth and development (Osmont et al. 2007; Petricka et al. 2012). Apart from the regulation of root elongation and branching, plant roots need to be able to adjust their growth direction in response to environmental signals, including gravity and water and nutrients availability (Potters et al. 2007; Pasternak et al. 2005). Previously studies showed that *Arabidopsis* roots grow straight down on

a vertical agar surface paralllel to the gravitational vector (Scherer and Pietrzyk 2014), and displayed a waving phenotype characterized by a sinusoidal growth pattern when grown on inclined agar gels (Thompson and Holbrook 2004). Gravitropism is confirmed to be an important component controlling root waving and the slanting behavior (Simmons et al. 1995). *Arabidopsis* ecotype Col-0 seedlings grow almost straight along the gravity vector on vertically placed 1/2 MS mediums (Migliaccio et al. 2013; Buer et al. 2000). Seedlings of Col-0 skew to the right when grown on vertical 1/2 MS medium under long light photoperiod (**Figure 1-3**) in our work, and the slanting angle was decreased when we changed the medium from basal MS to MS with vitamin, suggested that the medium type can alter direction growth of root and can even override the positive gravitropism growth behavior of vertically grown roots.

Root slanting and root waving are two processes that are regulated by the combined actions of gravitropism and/or the intrinsic circumnutation factor of root (Migliaccio et al. 2013). Root slanting in many *Arabidopsis* ecotypes always occurs in one direction only, to the right being the most common orientation (Simmons et al. 1995; Migliaccio and Piconese 2001), apparently resulting from the right-hand circumnutation of the root tip. Slanting is more pronounced at smaller inclinations, resulting in a clockwise coil on an almost horizontal agar surface due to right-hand circumutation and the perpendicular orientation of the gravitional vector. Here, we phenocopied the waving and slanting behavior of *Arabidopsis* root by growing wild type Columbia roots on tilted agar medium. We observed that Col-0 roots displayed less waving and a hooked (seemingly initiating the formation of a coil) root tip on an inclined agar plate at 45 ° (**Figure 3**), and making anticlockwise coils on the horizontal agar plates (roots that traversed across the agar surface even continued to make coils at the bottom of the petri dish). Thus, circumnutation is an intrinsic directional growth process in plant organs, but the torsion movementsof a root can be modulated by positive gravitropism and negative thigmotropism of root-gel interactions.

Sucrose affects root growth direction in *Arabidopsis thaliana*

Increasing the sucrose concentration not only promotes root growth and lateral root formation, but also modulates root directional growth of the primary root in *Arabidopsis* seedlings. In this present work, the rightward slanting of wild type and *rgir1* mutant seedlings was enhanced in the presence of sucrose (**Figure 2**), which is consistent with an earlier report by Buer's group (Buer et al. 2000). Subsequently, Buer et al. (2003) reported that ethylene modulates the root's waving/skewing response in a nutrient-dependent manner and clearly influenced the effects of sucrose on root skewing. In addition, cytokinin was identified to play a role in controlling direction/tropic responses including waving and producing coils and to interact with the ethylene, auxin, and glucose signaling pathways (Kushwah et al. 2011). In the presence of salt in the medium, sucrose also promotes the skewing without changing the growth direction (**Figure 5**), indicating that the sucrose-induced increased of the slanting angle in salt-treated roots is due to the increased growth rate.

The effects of sugars on plant growth and development are diverse, both as nutrient and as structural components (Rook et al. 2006; Lastdrager et al. 2014). In many cases, sugar itself acts as a signalling molecule in regulating it's own production and use in plant organs. Sugar sensing and signaling are involved in the entire plant life circle (Rolland et al. 2002; Zhou et al. 1998). High sugar accumulation may for a short period lead to arrest in *Arabidopsis* seedlings development, from which it can be recovered after transfer to soil without stress or to medium with an appropriated sugar concentration (Rolland et al. 2002; Lopez-Molina et al. 2001; Rook and Bevan 2003). However, sugar (i.e. sucrose and glucose) has marked and complex effects on the root system architecture and directional growth in plants, the mechanism of which is still not well understood. The study of sugar sensitive/insensitive mutants revealed that many of these mutants also have defects in hormone-synthesis or -signaling (Ljung et al. 2015; Rook et al. 2006; Zhou et al. 1998; Booker et al. 2010), hinting at a complex sugar-hormone-regulatory network that modulates various processes during plant growth and development.

Salinity effects on skewing phenotype of *Arabidopsis* root

Salinity is one of the major abiotic factors limiting crop yield worldwide (Vaughan et al. 2002). The root system of plants is the first and most important organ that senses salinity in the soil. Tolerance to salinity stress could partly be mediated by changes in the root system architecture, regulated through a complicated network of genes and proteins, and the relative levels of phytohormones (reviewed by Julkowska and Testerink 2015). Preliminary studies showed that mild salinity stress in the MS medium slightly suppressed primary and lateral root growth, while high level of salt (>100mM) were detrimental for root system development (See Chapter3, **Figure 2 and Figure 4**). In the present study, exposure roots of Columbia to 100 mM NaCl changed the skewing direction from right to left, consistent with obvious right-hand helical growth of epidermal root cells (**Figure 4 D**), in addition to root growth reduction and suppression of lateral roots (**Figure 4 B, C**). The response of Columbia roots growth to 50 mM NaCl was indistinguishable from those of roots cultured on the standard medium (with 1% Sucrose) without salt, but increasing the NaCl concentration to 100 mM caused the right-slanting angle to decreased to 0 °. Since neither the VGI of roots in wild type or *rgir1* mutant seedlings, nor the HGI was affected by equivalent concentrations of mannitol, the effect of 100 mM NaCl on growth direction must be due to the ionic effect and not to the increased osmolarity of the medium.

Potential roles for RGIR1 in root waving and skewing phenotype

A large collection of root waving and skewing mutants were identified during the screening of abnormal root growth behavior in recent years. The diversity of skewing mutants indicates that there must be a number of genes involved in the symmetry determination of root growth. One class of genes, involved in gravitropism, was shown to control auxin transport and responses, such as *aux1* (De Smet et al. 2007), *rgl1* (Simmons et al. 1995), *rha1* (Fortunati et al. 2008), and *knat1*

(Qi and Zheng 2013). Another group was shown to be involved in the arrangement of cortical microtubule including spiral genes (Shoji et al. 2006; Furutani et al. 2000), *sku6* (Sedbrook et al. 2004; Rutherford and Masson, 1996), and *WVD2* and *WDL1* (Yuen et al. 2003). Interestingly, CLE40, a protein functionally equivalent to the stem cell restricting CLV3, is required for normal root growth, and loss of CLE40 enhances root waving (Hobe et al. 2003). The wavy-root phenotype of plants with overexpression of CLE-like (CLELs) seems independent of the known environmental stimuli, which regulates root growth through an auxin-independent pathway (Meng et al. 2012), indicating the possibility of peptides that participates in intracellular signaling and in regulating root direction growth.

Based on an *in silico* analysis, five proteins (SPF1, T2E22.10, At23G47570, AT5G3310, and WAV2) are reported to be co-expressed with the *RGIR1* (At2g37050) gene, of which only *WAV2* is associated with RGIR1 according to co-localization. The roots of the *wav2* mutant bent with a larger curvature than those of wild type seedlings in wavy growth, as WAV2 reduces root bending induced by the environmental stimuli through inhibition of root tip rotation (Mochizuki et al. 2005). Moreover, the homolog of RGIR1 in rice plant (os0174550) was identified to be involved in the response to mannitol (Diervart et al. 2016). In the present work, we found that the waving and skewing patterns of *rgir1* mutants were indistinguishable from those of Columbia seedlings grown on an inclined agar plate, or when exposed to sucrose, salt or mannitol. Therefore, RGIR1 seems not involved in the response to mannitol and is not involved in the root growth direction.

Conclusion

Our observations demonstrate that roots display waving/skewing patterns and coils on tilted growth medium at different angles, and sucrose enhances the slanting angles of root at vertical plates by increasing the root growth rate. NaCl induces leftward skewing both at lower and higher concentration, and this salt-induced directional change is not due to the osmotic change alone. Although *rgir1-1* has a shorter main root length compared to wild type under optimal growth condition, root responses of *rgir1-1* seedlings are indistinguishable from wild type and *rgir1-2* seedlings in their skewing and waving behaviour under different treatments. Therefore, RGIR1 seems not to be involved in controlling the growth direction of root on the surface of agar.

Chapter6

General discussion

Role of RGIR1 in plant growth and development

Reverse genetics procedures are now well-established methods to identify the function of a gene. Analyzing the phenotypic characteristics caused by the mutation of a particular gene with inserted elements, such as T-DNA of *Agrobacterium* or a transposon (Bouché and Bouchez 2001; Feldmann 1991; Krysan et al. 1999) often give strong indications of what the specific role the gene plays. The result of the insertion of a T-DNA element in or near an *Arabidopsis*' gene depends on the place of insertion: the promoter, a coding region or a 3' un-translated region. In some cases, even a knockout mutant has no readily identifiable phenotype or displays a distinguishable phenotype compared to its wild type ecotype under the same growth conditions. The availability of large numbers of *Arabidopsis* T-DNA insertion lines has facilitated the discovery of functions of newly identified genes or proteins. Several steps are needed to characterize the phenotypic consequences of a particular T-DNA induced mutation. The lack of alteration of the phenotype could be caused by functional redundancy among members of a gene family and some other mutations have phenotypes that are conditional and can only be observed under specific physiological conditions.

Receptor-like kinases (RLKs) have emerged as a major component in the intercellular signaling processes within *Arabidopsis* root development (Wierzba and Tax 2013). Despite the large gene family of RLKs in the *Arabidopsis* genome, only a few of the total 610+ RLKs and RLPs have clear, identified, functions in mediating cell signaling during various stages of root development. Among these published RLKs that have a clear function in *Arabidopsis* root development, LRR-receptor kinases BRI1, BAK1, BRL1 and BRL3 reduce root length and root meristem size in a BR-dependent pathway (Caño-Delgado et al. 2004; Hacham et al. 2011; González-García et al. 2011). Besides interacting with BRI1 to control root growth via a BR-dependent pathway, SERKs were identified to interact with another unknown RLK, controlling root growth by regulating common target genes needed for root development (Du et al. 2012). In addition to BR, the transmembrane kinase TMK subfamily of RLKs show a reduced sensitivity to auxin and orchestrate plant growth by regulating cell expansion and cell division, and some of its members could also play a role in the auxin-mediated control of lateral roots development (Dai et al. 2013; Chang et al. 1992).

The Root-growth-inhibition-receptor 1 (RGIR1) belongs to the LRR I subfamily, with three conserved LRRs in the extracellular domain between the Malectin domain and the single transmembrane domain (Chapter 2). In the detailed root phenotypic analysis of two homozygous *RGIR1* alleles (*rgir1-1* and *rgir1-2*), the *rgir1-2* mutant, which is caused by the invalid insertion position close to 3'-untranslated region, did not exhibit visible changes under standard culture conditions when compared with it's wild type ecotype Col-0. However, the knock-out mutant allele *rgir1-1* has a shorter main root and less lateral roots when grown on agar medium under optimal growth condition (Chapter 2). Moreover, the transcripts of RGIR1 showed strong tissues specificity with higher expression in the root and lower in the rosette in the

reverse transcript analysis. Thus, RGIR1 only functions in the root system, while the shoot phenotype is not affected.

In Chapter 2, a significant reduction of seed size was observed in the *rgir1* mutant seeds compared with seeds of *Arabidopsis* ecotype Col-0, indicating a possible role for RGIR1 in controlling seed mass of plants. The seed germination process is affected strongly under low temperature and high salinity stresses, but no difference was found between wild type and mutant seeds under the identical conditions. However, the seeds of *rgir1-1* germinated earlier at high temperature than seeds of wild type and the *rgir1-2* mutant, suggesting a role for RGIR1 in the germination process. Germination is controlled by various environmental factors (and can be manipulated by hormonal treatment). Moreover, environmental factors can affect the endogenous factors that control germination (Bentsink and Koornneef 2008). Despite the smaller seed mass of *rgir1-1*, it exhibits a similar germination percentage as wild type under control condition and even a higher percentage when seeds were germinated under higher temperature of 25 ℃. Apparently, the germination process is not strongly positively correlated with the size of the dormant seed in our study.

In Chapter 3, the short root phenotype observed in *rgir1-1* mutant is always accompanied by a decrease of meristem size and elongation zone length and less cortex cells in the elongation zone. However, the average size of the cortex cells is not affected in the *rgir1-1* mutant root tip compared with ecotype Col-0. The development of the *Arabidopsis* root system is a dynamic process, comprised of diverse molecular mechanisms underlying different processes during root development that respond to both the external environment and the intrinsic signaling systems. Unlike those LRR-RLKs found in the hormone response pathways, RGIR1 may play a regulatory role in controlling root cell elongation and/or division under optimal growth condition and without any exogenous stress. Future work is needed to understand the regulatory process during root development and the regulatory mechanism of this newly identified LRR-RLK receptor.

Role of RGIR1 in response to diverse abiotic stresses

Plants are sessile organisms that have developed an extensive array of morphogenic responses when exposed to diverse abiotic stress conditions. Our detailed characterization of root system modification in *Arabidopsis* wild type (Col-0) under various abiotic stresses indicated that root elongation and root branching was distinctively affected by abiotic stresses (Chapter 4 and Chapter 5). Under abiotic stresses, results from the root trait of Col-0 and *rgir1* mutants (Chapter 3 and Chapter 4) indicate that abiotic stress imposed highly distinct effects on root growth and development of *Arabidopsis* plants. Main root length and lateral roots number of independent plants from Col-0, *rgir1-1*, and *rgir1-2* were selected in a Principle Component Analysis (PCA) to capture the major factor of RSA in response to abiotic stresses, including high/low temperature, salinity, osmotic stress, and plant hormone 24-EBL. As shown in **Figure 1A**, the two principal components accounting for 100% of the variation and the first principle component explained 95.2% of the

observed variation. Both PC1 and PC2 were related to main root length and lateral roots number of different genotypes and various abiotic stresses. The absolute value of Col-0, *rgir1-1*, and *rgir1-2* are close to 0 along PC1 axis indicating that the difference between genotypes is not significant. Despite the opposite direction on the PC1 axis, the absolute value for plant under treatment with 1 nM EBL or high temperature chamber (25 ℃) is similar with those under treatment with 100 mM salt, and low temperature chamber (15 ℃), approximately to 2. Thus, root growth and development is significantly increased under high temperature and hormone while it is severely prohibited under low temperature treatment and salinity (50 and 100 mM NaCl) (**Figure 1 B**). *Arabidopsis* plants under osmotic stress by supplied mannitol in the medium also showed decreased growth of main root and lateral development, but the inhibition was not as significant as salinity and low temperature stress.

Analysis of the kinetics of *rgir1-1* root growth

For the analysis of root growth methods have become available over the last couple of years that allow us to study the growth and development of roots at a high spatial resolution, enabling us to distinguish differences between the different processes that affect the length of roots. For *Arabidopsis* ecotypes, Beemster et al. (2002) used proxies for cell production and mature cell length to model the variation in root elongation rate. Starting from the clear premise that the rate of tip growth is the product of rate of cell production by divisions in the root meristem and the final cell length at the proximal end of the elongation zone (where the mature root zone starts, see **Figure 2**), the analysis of the different ecotypes resulted in large differences in both parameters (and also in the final outcome).

For our study it is interesting to determine what the reason for the shorter primary root in *rgir1-1* plants is: does the mutation affect the cell division rate or is the expansion of the cortical cells inhibited. This type of analysis would also make it an easy exercise to establish whether the processes leading to the short root phenotype of *rgir1-1*, are involved in other processes leading to a short root (i.e. abiotic stresses like low temperature or hormonal effects like ethylene). Using the root growth analysis program RootflowRT (see Chapter 3) the growth rate in the different root zones can be determined from series of root tip images taken 20 seconds apart. From the pixel (group) displacement the growth rate along the root tip is calculated. The growth patterns obtained resemble sigmoidal curves that can be fitted with a modified logistic growth function (**Figure 3**). The differences in overall growth rate of a root are identical to the maximal growth rate. The elongation zone is centered around the midpoint, the zone of the steepest increase in growth rate and the meristematic zone is represented by the zone where the growth rate is close to zero.

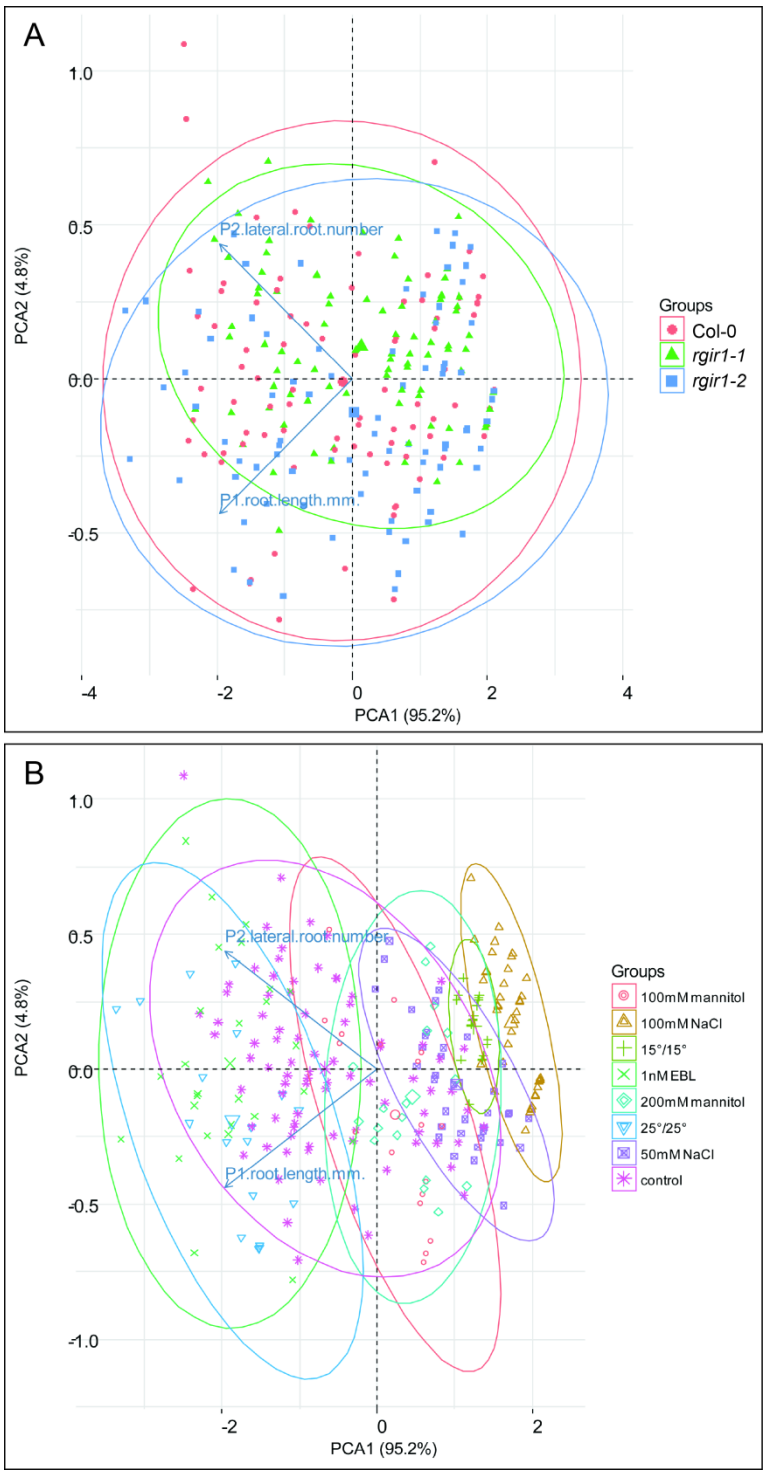

Figure 1. Biplot display of principal component analysis (PCA) based on the correlation of *Arabidopsis* ecotype Col-0 and two T-DNA insertion mutant lines in response to different abiotic stresses.

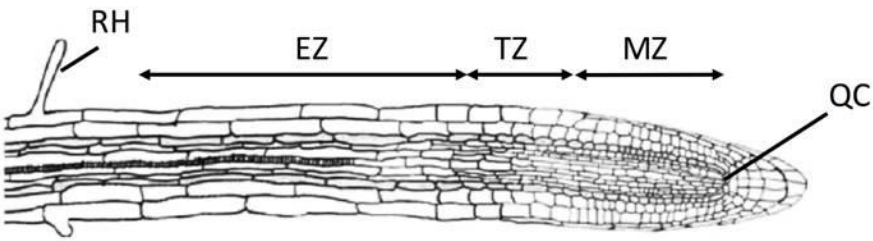

Figure 2. Schematic overview of the different zones in the root tip where cell division and cell expansion are mainly localized. The cortical cell files are derived from initials close to the quiescent center. Cell division (and limited cell elongation) continues in the meristem zone providing an ongoing "influx" of cells into the elongation zone, where cells no longer divide and most of the cell elongation takes place. Proximal of the elongation zone the cells no longer grow, the first root hairs are formed and the cell size has reached their maximal length (after Beemster et al. 2002).

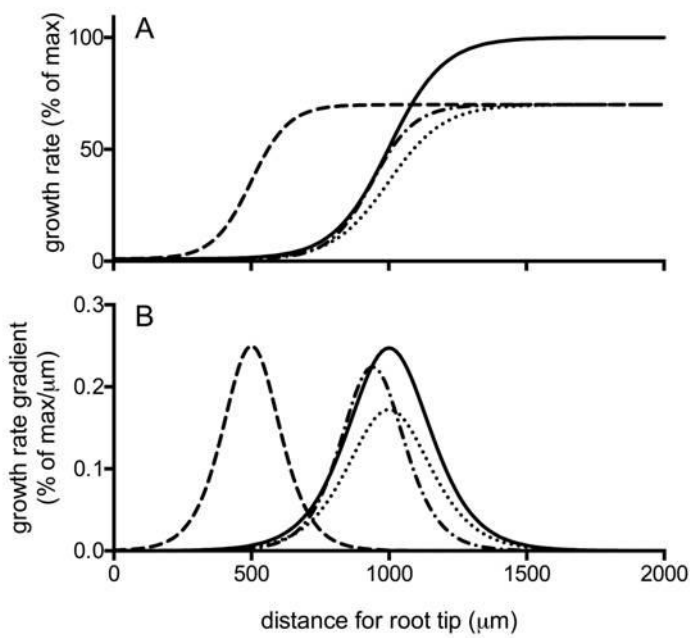

Figure 3. Family of theoretical curves for the growth rate along the root tip of roots with normal and reduced growth to 70%. A: Curves of the overall growth relative to the quiescent center (x=0) of normal growth (solid line), inhibited growth due to low expansion rate in elongation zone (dotted line), inhibited growth due to shortening of the elongation zone (dash-dotted line) and inhibited growth due to limited production of cells in the meristematic zone. **B**: Curves representing the first derivative of the growth rate curves shown in A.

In **Figure 3** the three basic processes that can lead to a reduced overall growth rate in root tips are illustrated. The first process is a reduction in the number of cells that

are available for elongation by limiting the rate of division in the meristematic zone. This will almost invariably result in a shift of the elongation zone towards the tip of the root. The resulting lines in the **Figure 4 A and B** are dashed. The second option is a reduction in the rate of cell elongation, the resulting mature cells that are on average shorter. While the elongation zone still has the same dimensions, the cell length remains shorter and the number cells still slowly expanding at any one time is increased. This model option is depicted by the lines that are dotted. The third option is a shortening of the expansion zone: the meristematic cells deliver cells to the elongation zone at the same rate, the elongation rate per cell is not lower, but the length of time the cells keep elongating is shortened. Again this will result in shorter mature cells in the cortex. As the results of Beemster et al. (2002) already indicated, combinations of modulating both cell production and cell size at maturity are both possible in realizing a certain root growth rate.

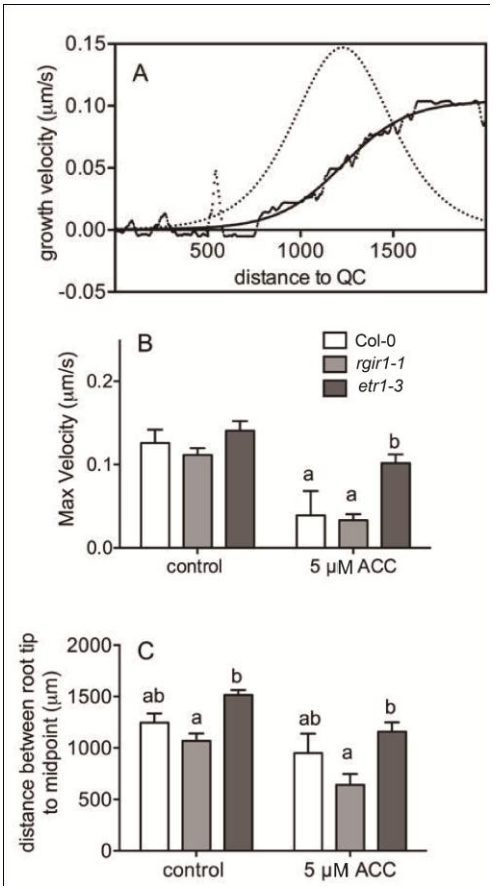

Figure 4. Comparison between the dynamic root growth parameters of wild type, *rgir1-1* and *etr1-3* roots on plates with control medium or medium supplemented with 5 µM ACC. A: The data resulting from the RootflowRT software fitted with a logistic growth curve (solid line). The first order derivative of the fitted line is also indicated (dotted line). Maximum velocity **(B)** and distance between root tip and midpoint **(C)** are derived from fitted logistic growth curves to the growth rate profiles.

If sufficiently high resolution data from the dynamic analysis of root growth profiles along the root tip could be obtained, distinguishing between the mechanism of root growth inhibition would indeed be possible. In **Figure 4** the results of a comparison between short roots phenotypes of *rgir1-1* and ACC-treated roots are shown. From the comparison between the genotypes wild type, *rgir1-1* and *etr1-3* (a mutant with a reduced sensitivity to ethylene) the slightly lower growth rate or *rgir1-1* and the slightly higher rate of *etr1-3* are noted. In both short phenotype situations (*rgir1-1* and exposure to ACC, a precursor of ethylene) it is clear that the lower growth rate correlates with a shift of the midpoint of the elongation zone towards the root tip. The only two model options that are compatible with this shift of the elongation zone are reduced cell production (reduced division rate in the meristematic zone) and the shortening of the elongations zone. When the length of the mature cells would be available for this experiment further distinction between these two models would be possible. From the ANOVA that was performed on these data it became clear that although the root length reduction in *rgir1-1* resembles that of ACC exposure, there was no statistically significant interaction between *rgir1-1* and ACC effects, which indicates that both mechanisms probably act independently.

Root waving and skewing behavior of Arabidopsis

Arabidopsis ecotype Columbia root exhibit a sinusoidal waving pattern and skew to one side of the plate when grown on inclined agar medium (Chapter 5). Since the discovery of waving and skewing root growth patterns, different models have been proposed to explain this surface-dependent root growth behavior (Roy and Bassham 2014). One widely accepted model explains waving and skewing as a result of intrinsic circummnutation, positive gravitropism and negative thigmotropism, while another interesting model considers the formation of these movements due to the physical interaction of the root tip with the medium. Other factors that have been implicated in regulating root movement on the surface of medium, including hormones and environmental cues (e.g. light, humidity, and nutrients in the medium). However, a unifying, generally accepted model for understanding the mechanism of waving and skewing movements is still lacking.

In the last decade, the isolation of wavy and skewing mutants has been used to discover genes implicated in skewing and waving behavior of the root (Oliva and Dunand 2007). Mutants with aberrant skewing phenotypes also have defects in the root cytoskeleton and cell wall modifications, indicating that function of genes affecting skewing are mainly involved in the re-arrangement of cytoskeleton and cell walls. The alteration of root skewing direction under high salinity and the enhanced skewing angle in the presence of sucrose (Chapter 5) suggests the processes that lead to the normal skewing response, based on the structure of the cytoskeletal elements, can interact with other signaling pathways and create flexibility in the root growth response to multiple environmental signals. The existence of mutations affecting skewing, but not waving, indicating that these two root growth behaviors are regulated by different processes. Compared with skewing, the process affecting waving is more complex and more difficult to explain since a range of different

factors is involved (Buer et al. 2003). Most discovered wavy mutants are defective in root gravitropism and show altered waving dynamics, such as amplitude and wavelength of waves, compared with wild type. Some mutants were identified involved in mediating the influx and efflux of the plant hormone auxin, which plays a fundamental role in the root gravitropic response. As lateral root initiation and the gravitropic response are both affected by the redistribution and transportation of auxin between different zones of the root tip, and lateral roots also space along the primary root in a regular left-right pattern that correlates with gravitropic response-mediated waves, this suggests that there is crosstalk between gravitropism, waving and lateral root formation.

The roots of the *wav2* mutant show wavy root growth with exaggerated curves compared to wild type *Arabidopsis*. WAV2 is probably a negative regulator root bending by inhibiting root tip rotation (Mochizuki et al. 2005). According to an *in silico* proteomic data analysis, RGIR1 is co-expressed with SPF1, T2E22.10, At3g47570, At5g43310 and associated with WAV2 based on co-localization, indicating a possible role for RGIR1 in controlling the waving and skewing phenotype of roots. However, the waving pattern of *rgir1* mutant was indistinguishable from that of Columbia seedlings grown on vertical medium plates or on the inclined plates either at 45 ° or 180 ° (Chapter 5). Therefore, we conclude that RGIR1 is involved in the regulation of root elongation, but that it has no effect on root directional growth on vertical agar medium.

Chapter7

Summary

Receptor-like kinases (RLKs) have emerged as major components in intercellular signaling during plant growth and development. Based on the similarity of the kinase domain sequences, the RLK family is comprised of more than 610 members, but for only a fraction we currently do know their function(s) during plant growth or in response to various abiotic and biotic stresses (Chapter1). In a previously study, one knock-out mutant of *Arabidopsis* gene At2g37050, here named *ROOT GROWTH INHIBITION RECEPTOR1* (*RGIR1*), displayed a distinctly shorter primary root and less lateral roots. The *RGIR1* gene encodes a protein of 934 amino acids with a predicted molecular mass of 103.4 kD, which belongs to the LRR-I transmembrane receptor-like protein kinase family, with three conserved LRRs in the extracellular domain. Since most work published for RGIR1 was mainly focused on comparison of transcriptome analyses under particular stress conditions, the role of RGIR1 in plant growth and development was still lacking.

In Chapter2 seed germination of two T-DNA insertion mutants (*rgir1-1* and *rgir1-2*) under cold and salt stress was studied and a detailed screen for alteration of their root system architecture under optimal growth condition was performed. Seed germination was strongly affected by low temperature and salinity treatment both for wild type and mutants. Whereas mutant seeds have a smaller seed size compared with wild type, no evidence was found for a direct link between RGIR1 with control of seed size and seed germination. Seedlings of *rgir1-1* mutants showed a shorter main root length and smaller root surface area on agar plates, while the leaf phenotype was not affected on agar or in soil at optimal temperature, indicating that RGIR1 only has a role in root development.

In Chapter3 root morphology, to quantify cell number and size, and kinematic parameters of root elongation, to establish the location, size and activity of the elongation zone, in wild type and *rgri1-1* root tips were determined under optimal condition and when exposed to cold or salinity stress. In the presence of salt or cold stress, root growth and development were strongly affected with shorter main root length and less lateral roots in Col-0 and *rgir1-1*mutant. The shorter root phenotype of *rgir1-1* seedlings is associated with a lower elongation rate and decreased cortex cell number in the transition zone and elongation zone of the root tip under optimal growth condition. Differences in main root and lateral roots between *rgir1-1* and wild type disappeared when exposed to cold and salinity, but were more pronounced at high temperature, indicating that RGIR1 is a positive regulator in the process of root growth and development under optimal growth condition. The pathways for RGIR1 controlling root elongation seems to be independent of those involved in the responses to cold and salinity.

In Chapter4 effects of culturing conditions on root growth patterns in Col-0 and *rgir1* mutants were studied. Sucrose (1.5%) induces a waving and skewing phenotype on hard (1.5% micro-agar) medium, and strongly reduced lateral roots formation and an increase length of the main root in both Col-0 wild type and *rgir1* mutants. Main root growth and root branching in wild type and mutants were inhibited under salt or osmotic stress, and at low pH. Sulfur-deficiency didn't affect

main root growth of Col-0 seedlings but lateral roots formation was strongly stimulated compared with those grown on sulfur-sufficient medium. More lateral roots developed in *rgir1-1* roots than on Col-0 roots, grown on the same sulfur-deficiency medium, indicating a possible role for RGIR1 in the process of lateral root initiation or emergency.

In Chapter 5 we studied the effects of salt stress and root-agar interaction on root skewing behavior in *Arabidopsis thaliana*. Root of Col-0 displayed rightward slanting on vertically placed agar medium, and this slanted phenotype was enhanced in the presence of 1% sucrose. Anti-clockwise root coils and hooked root tips were identified in Col-0 seedlings when the agar plates were placed horizontally or inclined at an angle of 45 °, respectively. High salinity altered root skewing direction, combined with severe suppression of main root elongation and lateral roots formation in Col-0. These responses were observed when exposed to NaCl, but not under osmotic stress. Roots of *rgir1-1* seedlings responded the same as wild type, both on inclined agar medium and on vertical medium with high salt or mannitol, indicating that RGIR1 has a role controlling root elongation, but is not involved in the directional growth of the root tip.

In summary, it can be concluded (Chapter 6) that RGIR1 is an LRR-I receptor-like kinase which does have a function in the root system architecture of *Arabidopsis*. However, changes in root morphology induced by high or low temperature or the hormone EBL, or exposure to salinity, are not affected by mutating the RGIR1 gene. The dynamic analysis of the root growth profile along the root tip in *rgir1-1* and *etr1-3* (a mutant also with shorter main root length and reduced sensitivity to ACC), does not show a statistically significant interaction between *rgir1-1* and ACC treatment, thus, both mechanisms controlling the reduction of root length probably act independently. Although RGIR1 is associated with WAV2, which shows wavy root growth and exaggerated curves compared to *Arabidopsis* wild type, no evidence was found for RGIR1 in controlling the directional growth of root in this thesis.

Chapter8

Samenvatting

Receptor-like kinases (RLK's) zijn belangrijke componenten in de intercellulaire signaaloverdrachtin zowel dieren als planten en spelen een belangrijke rol in groei en ontwikkeling. Op basis van overeenkomsten in de de kinase domein sequenties onderscheiden we 610 eiwitten die tot de de RLK familie behoren.Van slechts een klein deel van deze 610 RLK's weten we de precieze functie tijdens de groei van planten of in hun reactie op verschillende abiotische / biotische stress omstandigheden (hoofdstuk 1). In een eerder onderzoek werd een mutantge ïdentificeerd (At2g37050, in dit proefschrift ROOT GROWTH INHIBITION RECEPTOR1 (RGIR1) genoemd), die een duidelijke kortere primaire wortel had en ook minder zijwortels vertoonde.Het RGIR1 gen codeert voor een eiwit van 934 aminozuren met een voorspelde moleculaire massa van 103,4 kD, dat behoort tot de LRR-I transmembrane receptor-like kinase familie, met drie geconserveerde leucine-rich repeats (LRRs) in het extracellulaire domein. Aangezien het meeste dat al bekend was over RGIR1 voornamelijk was bebaseerd op vergelijking van genexpressieonder verschillendestressomstandigheden, ontbrak een duidelijke rol voor het RGIR1 gen.

In hoofdstuk 2 worden zaadkiemingsexperimentan van twee T-DNA insertiemutanten van RGIR1 (rgir1-1 en rgir1-2) onder lage temperatuur en bij blootstelling aan een hoge zoutconcentratie beschreven. Tevens werd een gedetailleerd analyse gemaakt van de verandering in de structuur van wortelstelsel van RGIR1 mutanten ten opzichte van wildtype planten.De zaadkieming werd sterk be ïnvloed door een lage temperatuur en door een hoog zoutgehalte, zowel voor wildtype als mutanten. Terwijl de zaden van mutantenwel kleiner zijndan die van het wildtype, werd geen bewijs gevonden dat RGIR1 een rol heeft in de controle op zaadgrootte en -vorming. Zaailingen van rgir1-1 mutant vertoonden een kortere lengte van de primaire wortel en een kleiner worteloppervlakte, maar de ontwikkeling van de spruit of de bladeren was identiek in wildtype en mutanten, wat impliceert dat RGIR1 alleen een rol heeft in de ontwikkeling van de wortel.

In hoofdstuk 3 werden morfologie (celgroote en –aantal) en kinematische parameters (locatie, omvang en activiteit van de groeizone) van de worteltop gemeten in de wildtype en rgri1-1 planten onderzowel optimale omstandigheden, lage temperatuur en hoge zoutconcentratie. In aanwezigheid van zout of bij lage temperatuur werden wortelgroei en -ontwikkeling sterk be ïnvloed, resulterend in een kortere hoofdwortellengte en minder zijwortels in Col-0 en rgir1-1 mutant. Het kortere wortelfenotype van rgir1-1-zaailing is geassocieerd met een lagere celstrekking en een lager aantalcortexcellen in de overgangszone en de strekkingszonevan de wortel onder optimale groeiconditie. Verschillen tussen rgir1-1 en wildtype inwortellengte en aantallen laterale wortels verdwenen wanneer de planten werden blootgesteld aan koude en zoutg, maar werden meer uitgesproken bij hoge temperaturen.Dit duidt erop dat RGIR1 een positieve regulator is van het proces van wortelgroei en –ontwikkeling onder optimale omstandigheden. Het regulatiemechanismewaarmee RGIR1 de wortellengte beinvloedt, lijkt onafhankelijk het mechanisme waarmee lage temperatuur en zout de wortellengte veranderen.

In hoofdstuk 4 werden effecten van groeiomstandighedenop de structuur van het wortelstelvan van Col-0 en rgir1 mutanten bestudeerd. Sucrose (1.5%) induceert een golvend en 'scheef'fenotype op hard (1,5% micro-agar) medium, een sterk verminderde aantal laterale wortels en een langere hoofdwortel in zowel wildtype (Col-0), als rgir1 mutant planten. Blootstelling aan zout, een lage pH, of een hoge osmotische waarde remt de lengte groei en de ontwikkeling van zijwortels in zowel wildtype als in de mutanten. Zwavel-deficiëntie had geen invloed op de lengte van de hoofdwortel van Col-0-zaailingen, maar de vorming van laterale wortels werd sterk gestimuleerd in vergelijking met de planten opmedium met voldoende zwavel. Laterale wortels van rgir1-1 zaailingen waren sterker gestimuleerd dan die van wildtype planten op hetzelfde zwavel-deficiëntie medium, wat mogelijke duidt op een rol voor RGIR1 in de intiatie van laterale wortels.

In hoofdstuk 5 bestuderen we de effecten van zoutstress en de interactie van de wortel met het oppervlakte van agarmediumop het wortelfenotype van *Arabidopsis thaliana*. Wortels van Col-0 op verticaal geplaatste agar platen hebben een duidelijk neiging om scheef naar rechts te groeien, en dit schuine fenotype wordt versterkt in aanwezigheid van 1% sucrose. Op platen die horizontaal of onder een hoek van 45 graden worden geplaatst vertone een wortels een ant-iclockwise kurketrekker-achtige groei waarbij de wortelpunt een krul vertoont.Een hoog zoutgehalte verandert de groeirichting van de wortelpunt, terwijl tegelijkertijd de lengtegroei van de hoofdwortel en de ontwikkeling van zijwortels sterk wordt geremd in Col-0. Deze remming wordt veroorzaakt door NaCl, en niet door de hogere osmotische waarde. Wortels van rgir1-1 mutant zaailingen reageerden op een vergelijkbare manier: op vertikaal geplaatste platen en onder NaCl en osmotische (mannitol) stress reageert rgir-1 zoals wildtype. Dit lijkt erop te wijzen dat RGIR1 alleen een rol heeft bij de lengtegroeivan de wortel, maar niet op de groeirichting van de wortelpunt.

Samenvattend kan worden geconcludeerd (hoofdstuk 6) dat RGIR1 een LRR-I receptor-likekinase is diealleen in de ontwikkeling van de wortel van Arabidopsis een rol van betekenis speelt en geen effect heeft op de ontwikkeling van de spruit of van het blad. Wortelgroei en -ontwikkeling wordt aanzienlijk verhoogd onder hoge temperatuur en behandleing met het hormoonEBL, terwijl lage temperatuur en een hoog zoutgehalte wortelgroei remmen, in zowel wildtype en mutanten. RGIR1 lijkt geen functie te hebben in de respons op deze abiotische stressen en hormoonbehandeling. Uit de dynamische analyse van het wortelgroei profiel langs de wortelpunt in rgir1-1 en etr1-3 (een mutant ook met kortere wortellengte en verminderde gevoeligheid voor ACC), waren er geen statistisch significante interacties in rgir1-1 en ACC behandeling, dus beide mechanismen die de vermindering van de wortellengte beinvloeden, treden onafhankelijk op. Hoewel RGIR1 is geassocieerd met WAV2, die een golvende wortelgroei en overdreven wortelcurvatuur veroorzaakt in vergelijking met Arabidopsis wildtype, werdin dit proefschriftgeen bewijs gevonden voor een rol van RGIR1 de regulatie van de richtinggroei van de wortelpunt.

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Abbreviation

ACR4	*Arabidopsis thaliana* homologue of CR4
AD	apical domain
ALE1	ABNORMAL LEAF SHAPE1
ALE2	ABNORMAL LEAF SHAPE2
BAK1	BRI1 ASSOCIATED KINASE1
BAM1/2	BARELY ANY MERISTEM 1/2
bHLH	basic helix-loop-helix
BL	brassinolide
BR	brassinosteroid
BRI1	BRASSINOSTEROID INSENSITIVE1
BRL1	BRI1-LIKE1
BRL3	BRI1-LIKE1
BSKs	BR-signaling kinases
CD	central domain
CFRs	cell file rotations
CLE	CLAVATA/ENDOESPERM SURROUNDING REIGON
CLV1	CLAVATA1
CPC	CAPRICE
CR4	CRINKLY4
CRF2	CYTOKININ RESPONSE FACTOR 2
CRLK1	Calcium/CAM-regulated RLK
CRPs	cysteine-rich peptides
CSCs	columella stem cells
CWR	cell-wall-remodelling
CZ	central zone
EBL	epi-Brassinolide
EGL3	ENHANCER OF GLABRA3
EPF	Epidermal Patterning Factor
ER	ERECTA
ERL1	ERECTA-like1
ERL2	ERECTA-like2
ETC1	ENHANCER OF TRY AND CPC

FER	FERONIA
FLS2	FLAGELLIN-SENSITIVE2
GFP	green fluorescent protein
GHR1	GUARD CELL HYDROGEN PEROXIDE-RESISTANT1
GMC	guard mother cell
GSO1	GASSHO1
GSO2	GASSHO2
HAE	HAESA
HGI	horizontal growth index
HSL2	HAE-LIKE2
IDA	INFLORESCENCE DEFICIENT IN ABSCISSION
LRPs	lateral root primordias
LRRs	leucine-rich repeats
LRs	lateral roots
MAMP	microbe-associated molecular pattern
MAP	MITOGEN-ACTIVATED PROTEIN
MOL1	MORE LATERAL GROWTH1
NPA	naphthylphthalamic acid
OC	organizing center
OZ	organizing zone
PBS1	AVRPPHB SUSCEPTIBLE1
PEPR1	PEP1 RECEPTOR1
PERK	proline-rich extension-like receptor kinase
PI	Propidium Iodide
PLT	PLETHORA
PR	primary root
PXY/TDR	PHLOEM INTERCALATED WITH XYLEM/TDIF RECEPTOR
PZ	peripheral zone
QC	quiescent center
QTL	quantitative trait locus
RAM	root apical meristem
REGR	relative elemental growth rate

RGIR1	Root Growth Inhibition Receptor
RHs	root hairs
RLCK	receptor like-cytoplasmic kinase
RLKs	Receptor-like kinases
RLPs	receptor-like proteins
ROS	Reactive oxygen species
RPK1	RECEPTOR PROTEIN KINASE1
RPK2	RECEPTOR-LIKE PROTEIN KINASE2
RSA	root system architecture
RTKs	receptor tyrosine kinases
RUL1	REDUCED IN LATERAL GROWTH1
RZ	rib zone
SAM	shoot apical meristem
SCAREROW	WUSCHEL-RELATED HOMEOBOX5
SCM	SCRAMBLED
SCR	SCAREROW
SERK1/2	SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1/2
SHR	SGORT ROOT
SIMR	stress-induced morphogenic response
SOS2	Salt Overly Sensitive 2
SPCH	SPEECHLESS
SSP	SHORT SUSPENSOR
TDIF	TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR
TMK	TRANSMEMBRANE KINASE
TMM	TOO MANY MOUTHS
TOAD2	TOADSTOOL2
TRY	TRIPTYCHON
TTG	TRANSPARENT TESTA GLABRA
VGI	vertical growth index
WAKs	WALL-ASSOCIATED KINASEs
WER	WEREWOLF

WOX5	WUSCHEL-RELATED HOMEOBOX5
WUS	WUSCHEL
XIP1	XYLEM INTERMIEXD WITH PHLOEM1
YDA	YODA
ZmPK1	the maize putative protein kinase-encoding cDNA clones

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